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UTILIZATION OF CASSAVA AND CASSAVA WASTE THROUGH FERMENTATION TECHNOLOGY

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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SUBMITTED BY

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PROJECT LEADER

Department of Microbiology

KASETSART UNIVERSITY

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Raw Starch Digestion by *Rhizopus* spp.

Introduction

As previously reported (Progress report, June-November 1975) a strain of *Aspergillus* sp. and two of *Rhisopus* were selected basing on their efficiency in raw starch digestion. Although enzymes produced by these selected strains were not significantly different in terms of activity but the strains were found to be competitive over each others in some other properties. While the *Aspergillus* was considered superior regarding on the optimal pH of the enzyme activity which was found to be in the lower range than those of the *Rhizopus*, the latters were found to be more tolerant to condition limitting of oxygen and high temperature. However, the results obtained in the last reporting period was not conclusive enough for making a final selection of the most appropriate strain to be used for enzyme preparation. In this reporting period, optimization on culture conditions for maximal enzyme production of the *Rhizopus* spp. was made. Some physicochemical properties were also determined, so that the strains can be compared under their optimal conditions.

Materials and Methods

Microorganisms: *Rhizopus* sp. 102 R and *Rhizopus* sp. N37 were grown on starch agar slant for 5 days and kept for stock

cultures. The culture was transferred to potato dextrose agar at time of study.

Preparation of crude enzyme: Solid state culture was carried in an autoclavable plastic bag (Lotong and Suwanart, 1983). Unless stated, the sterilized substrate was prepared using 18 g wheat bran (11% moisture content), 2 g rice husk and 20 ml distilled water. Initial pH of the substrate so prepared is 5.4-5.5. To each bag, 1 ml of spore suspension containing ca. 10^5 spore/ml was inoculated. The culture was incubated at 35°C for 48 hours. To each koji bag, 150 ml distilled water containg 0.5 ml toluene was added. The koji was broken and thoroughly mixed by external marceration of the bag. The mixture was then transferred to a 500 ml flask and shaked in a water bath shaker where temperature was controlled at 4°C for 2 hours. The liquid collected after filtering was centrifuged at 100,000 rpm for 15 minutes. The supernatant was used as crude enzyme extract.

Determination of raw starch digesting activity: The reaction mixture containing 0.1 g raw cassava starch (preheated at 100°C overnight), 3 ml distilled water, 1 ml 0.1 M citrate buffer (pH 4.0), 1 ml crude enzyme extract and was shaken at 150 rpm/min at 35°C for 48 hours. The hydrolysate was determined for glucose using glucose oxidase method. Amount of liberated sugar was obtained by subtraction of the total concentration by that originally containing in the enzyme extract.

Results and Discussions

Optimization of culture conditions of *Rhizopus* spp. for production of raw starch digesting enzyme:

To establish an optimal culture condition for enzyme production, the following parameters were studied: initial pH, incubating temperature and moisture content of the substrate.

Initial pH: Results as shown in Table 1 and Fig. 1, 2 revealed that both strains of the *Rhizopus* spp. produced highest enzyme activity at pH 5.5 which was slightly higher than that optimal for *Aspergillus* J8 previously reported. However, when comparing between the two *Rhizopus* strains, N37 was found to be more competitive than strain 102R at lower pH. It was also shown in Fig. 1 and 2 that at pH 3.5, the enzyme produced by the former was 16% less active than that obtained at the optimal pH, whereas the enzyme produced by the latter was 27% less active.

Incubating temperature: Table 2 and Fig. 3, 4 indicated that strain N37 was comparatively tolerant to high temperature than strain 102R. It was observed that at temperature ranging from 35 to 40°C, the enzymes produced by the former were not significantly different in activity, while that produced at 40°C by the latter was less active.

Table 1. Effect of pH on production of raw starch digesting enzymes by *Rhizopus* sp.

strain No. of *Rhizopus* sp	pH	glucose liberated (mg %)
N_{37}	3.5	2219.4
	4.0	2330.7
	4.5	2324.1
	5.0	2458.3
	5.5	2668.2
	6.0	2507.9
	6.5	2454.5
102 R	3.5	2000.01
	4.0	2297.3
	4.5	2391.3
	5.0	2539.2
	5.5	2705.9
	6.0	2542.7
	6.5	2540.3

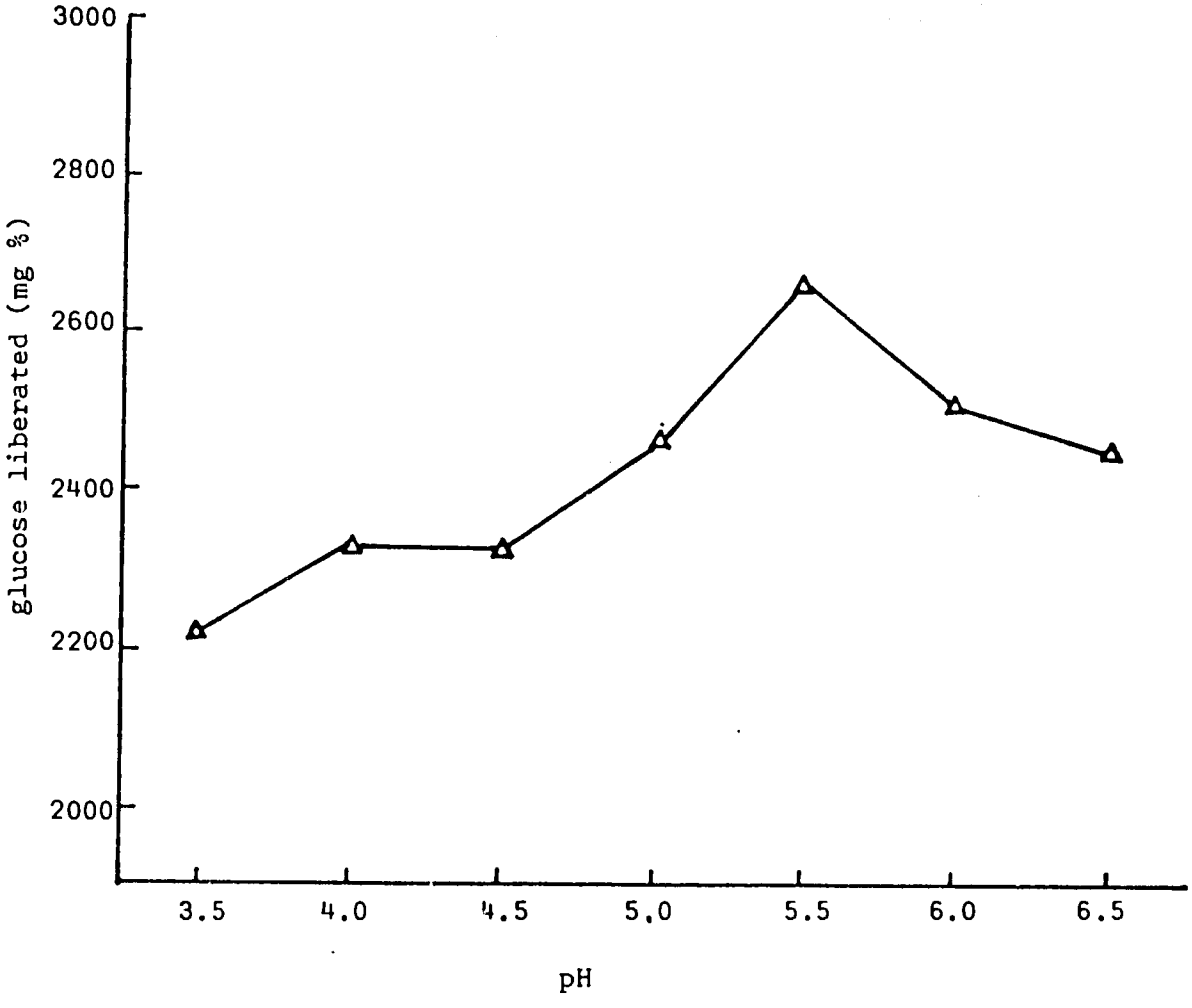


Fig 1. Effect of pH on production of raw starch digesting enzymes by Rhizopus sp. N37

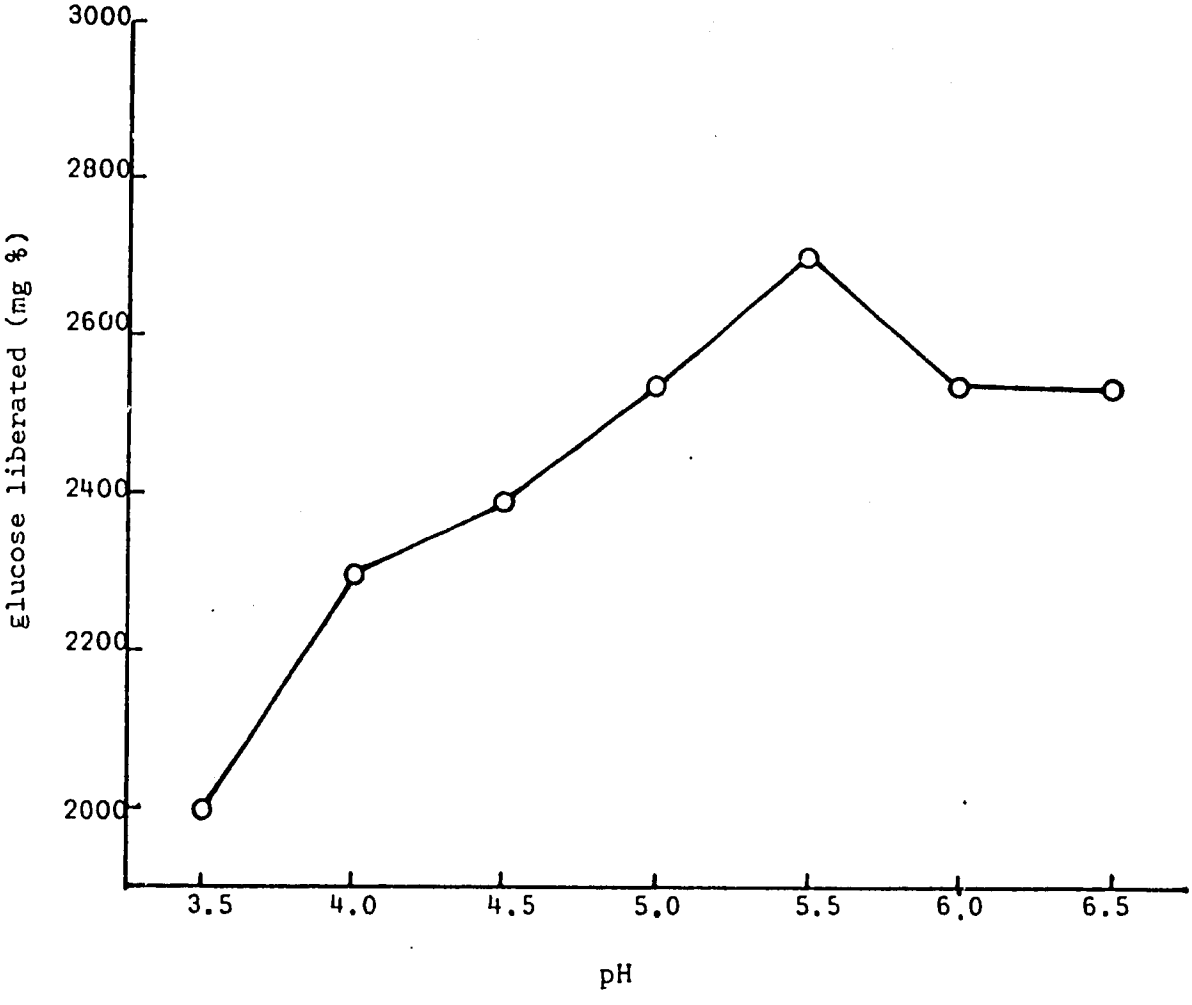


Fig 2. Effect of pH on production of raw starch digesting enzymes by Rhizopus sp. 102 R.

Table 2. Effect of temperature on production of raw starch digesting enzymes by *Rhizopus* sp

Rhizopus sp. Strain No.	Temperature °C	glucose liberated (mg %)	unit enzyme g Koji wet wt
N_{37}	35	2267	29.5
	37	2197	28.6
	40	1948	25.4
	42	1388	18.1
102 R	35	2095	27.3
	37	2386	31.9
	40	1775	23.1
	42	544	7.1

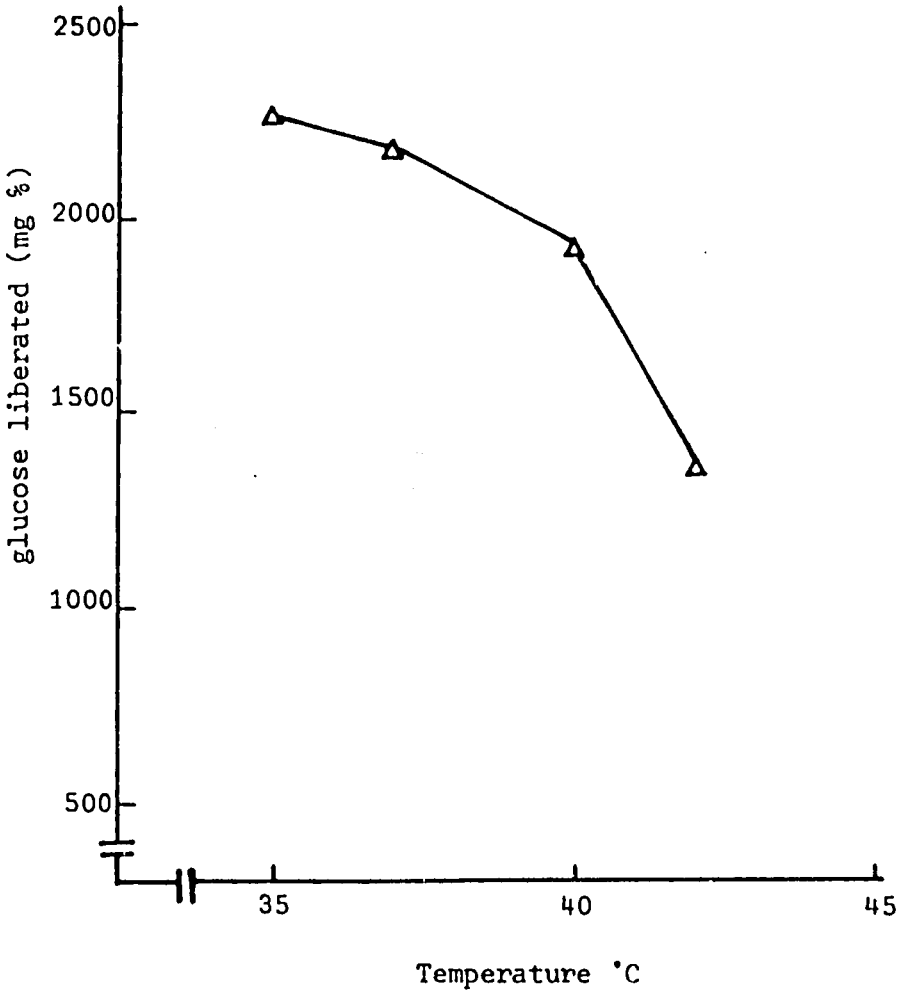


Fig 3. Effect of temperature on production of raw starch digesting enzymes by Rhizopus sp N_{37}

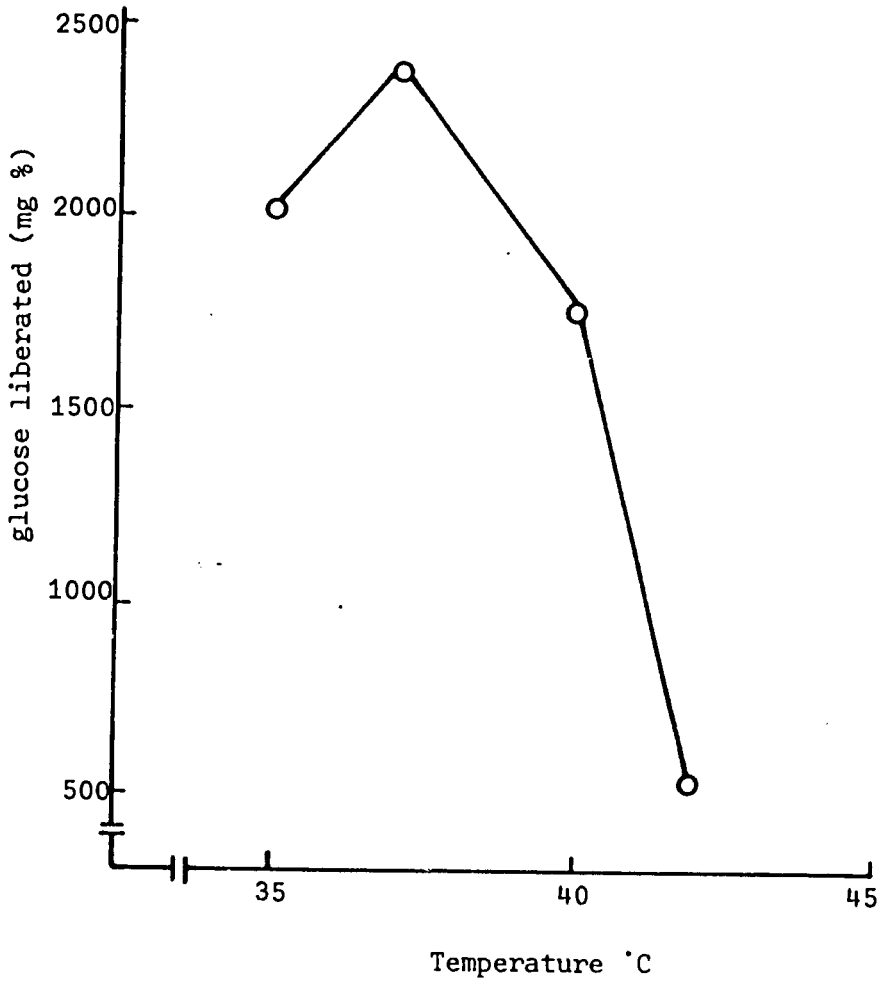


Fig 4. Effect of temperature on production of raw starch digesting enzymes by Rhizopus sp. 102 R.

Initial moisture content: Results as shown in Fig. 5 ,6 show that strain N37 had a narrow range of optimum moisture content (47-50%). Strain 102R although required higher moisture for maximum enzyme production, comparable activity was observed in a wider moisture range (50-60%).

Optimum conditions of enzyme reaction on raw starch digestion:

Culture was grown under conditions optimal for enzyme production as previously mentioned. Substrate containing 9 g wheat bran, 1 g rice husk and 9 ml water (50% moisture content) and pH adjusted to 5.5 was used. Crude enzyme extract obtained from culture incubated at 35°C for 48 hours was determined for some physicochemical properties.

Optimum pH: Tables 3, 4 and Fig. 7, 8 suggested that enzymes of both strains were most active at pH 4.5. It was also shown that the enzyme of strain N37 had a wider range of effective pH than that of strain 102R. While the former gave only 5 and 9% less active than its optimal pH, the latter was found to be 12 and 16% less active.

Optimum temperature: Raw starch digestion by the crude enzyme was carried out at various temperatures, ranging from 35-50°C. The effective temperature of the enzyme of strain N37 was observed in the range of 40-50°C and was most active at 45°C

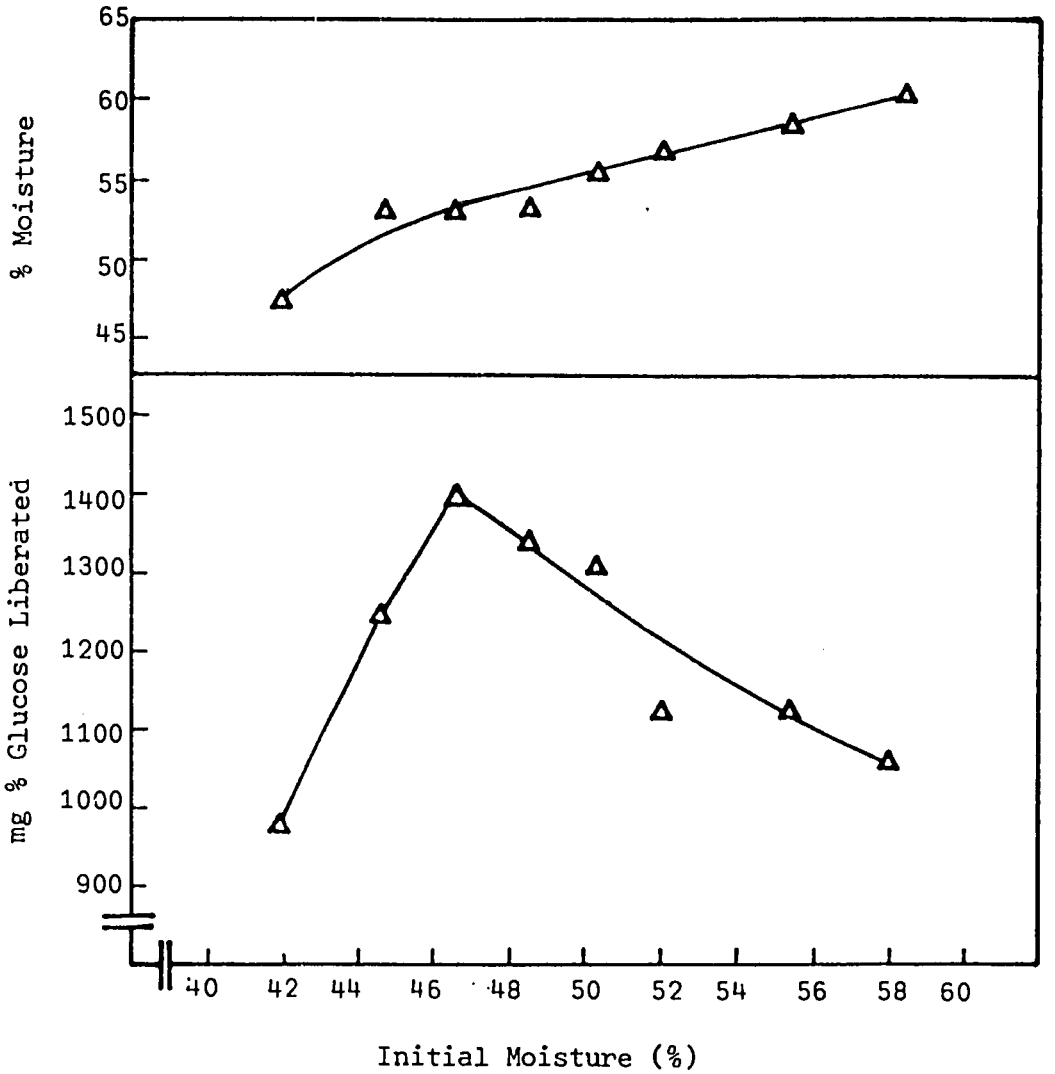


Fig 5. Effects of moisture content on porduction of raw starch digesting enzyme of Rhizopus sp. N_{37} using wheat bran as substrate

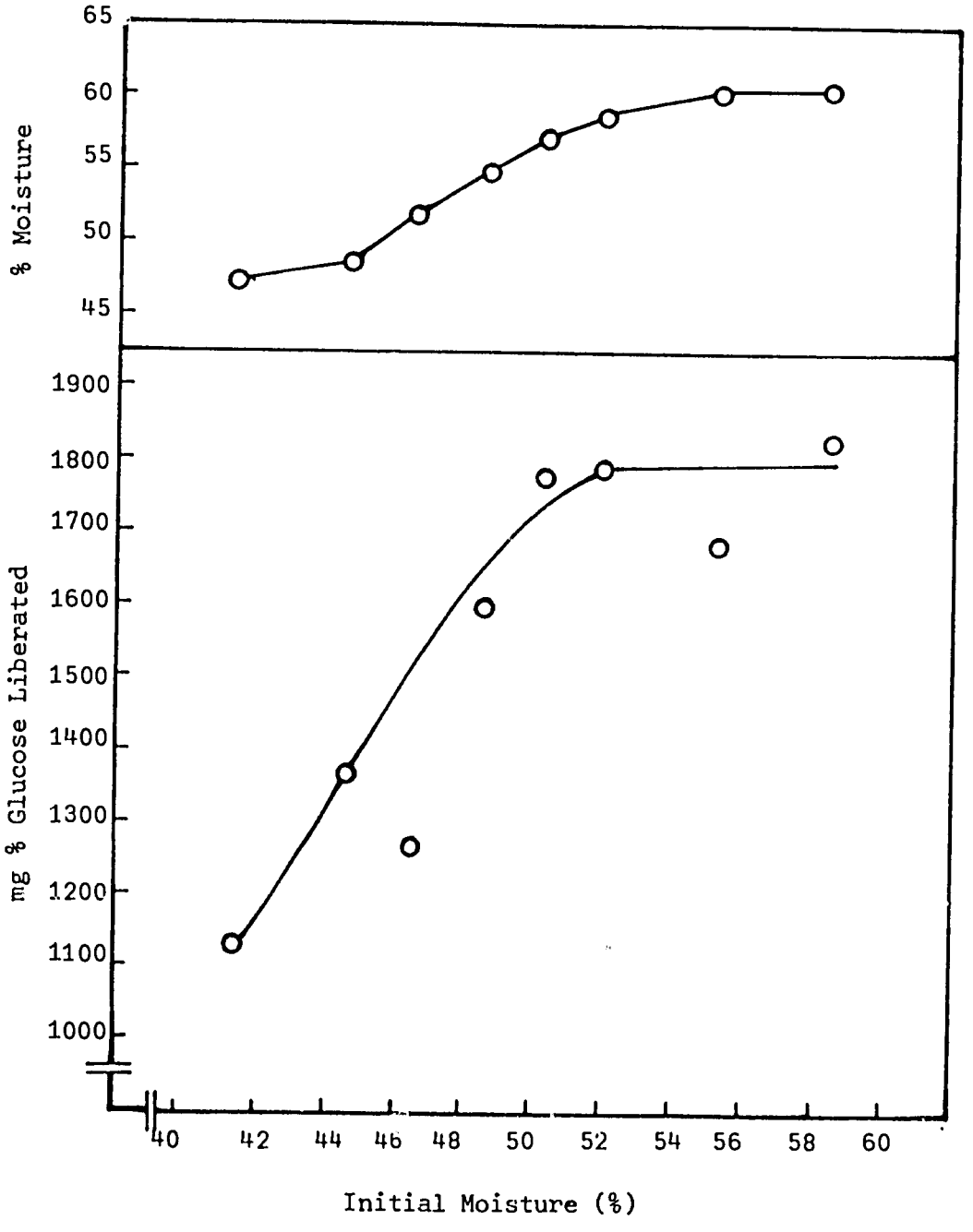


Fig 6. Effects of moisture content on production of raw starch digesting enzymes of Rhizopus sp. 102 R using wheat bran as substrate.

Table 3. Effect of pH on raw starch digesting activity of crude enzyme extract of Rhizopus sp. N_{37}

pH	glucose liberated (mg %)	Relative activity
3.5	1430	62.1
4.0	2182	94.8
4.5	2302	100.0
5.0	2082	90.4

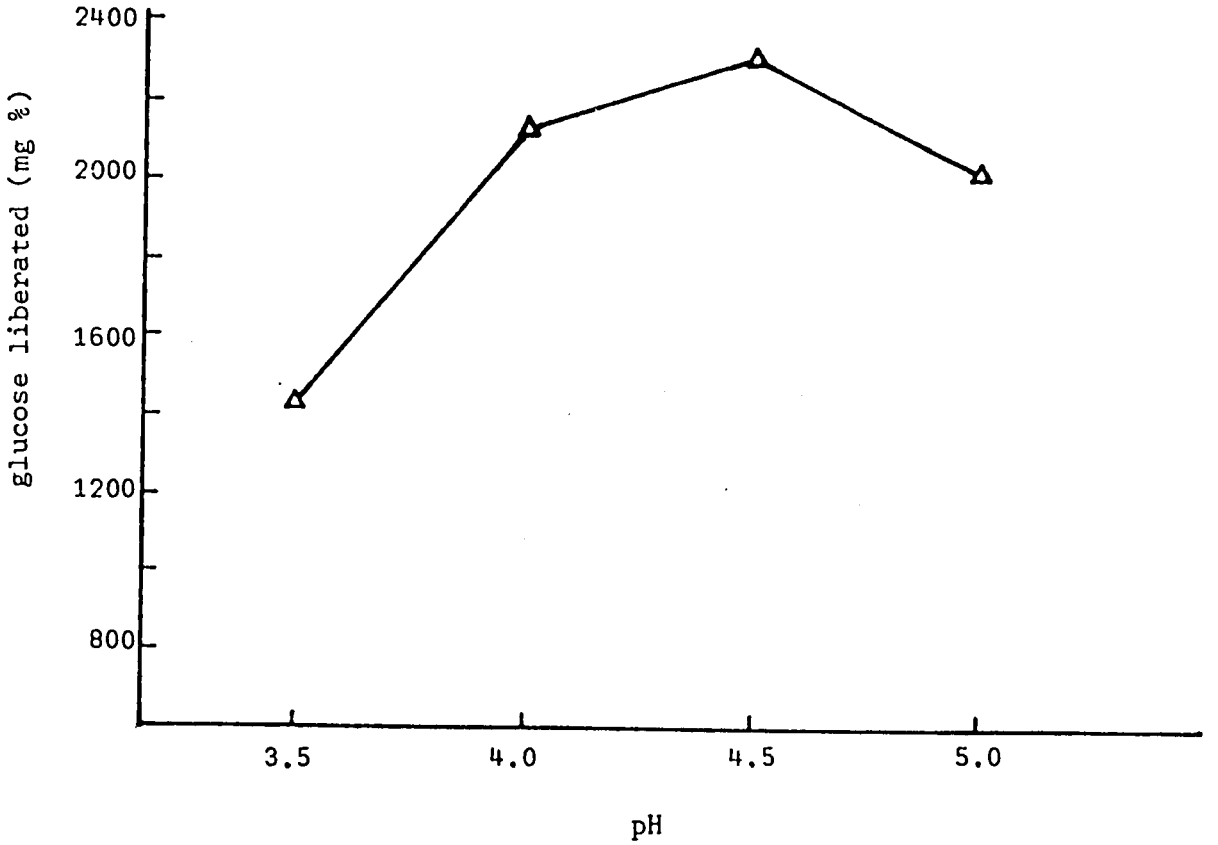


Fig 7. Effect of pH on raw starch digesting activity of crude enzyme extract of Rhizopus sp. N_{37}

Table 4. Effect of pH on raw starch digesting activity of crude enzyme extract of *Rhizopus* sp. 102 R

pH	glucose liberated (mg %)	Relative activity
3.5	957	40.9
4.0	1770	75.6
4.5	2341	100.0
5.0	1972	84.2

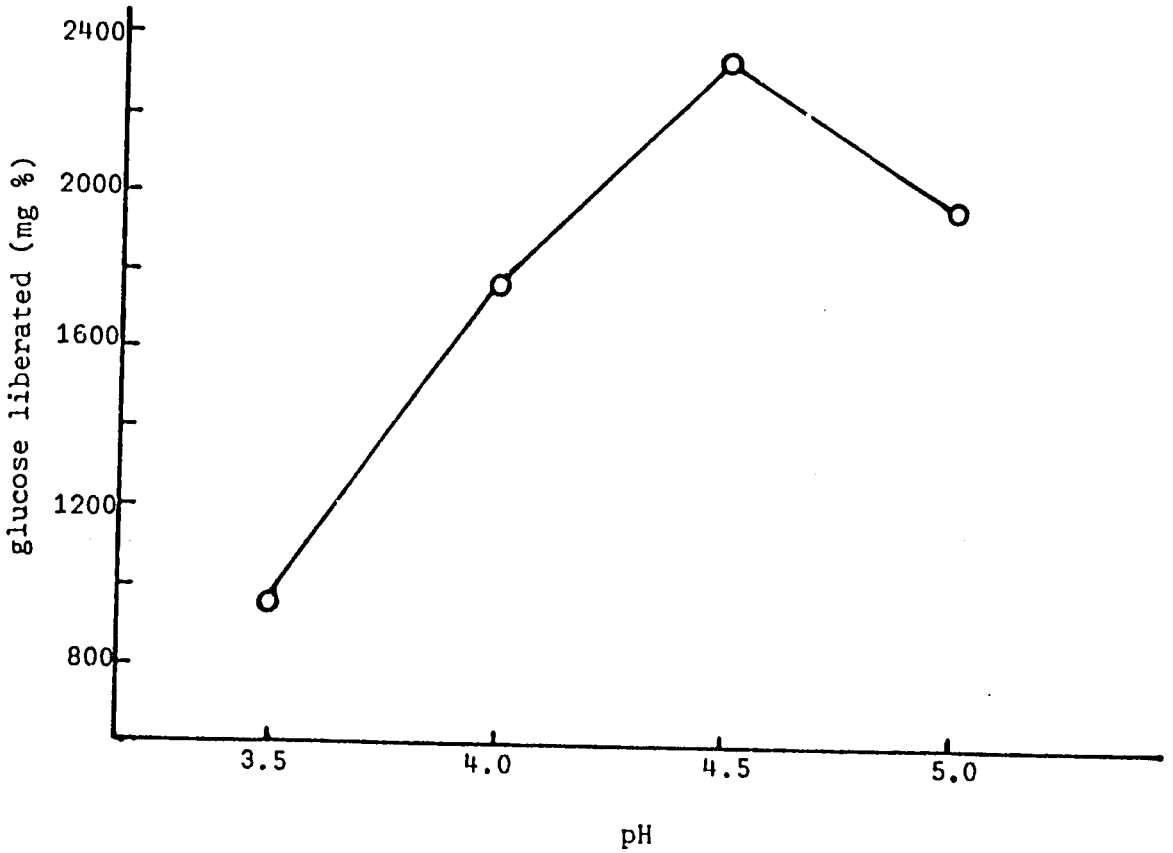


Fig 8. Effect of pH on raw starch digesting activity of crude enzyme extract of Rhizopus sp. 102 R

(Table 5, Fig. 9). Table 6 and Fig 10 show that the enzyme of strain 102R had optimum temperature at 40°C. It was also likely that its effective temperature was not as broad as that of strain N37. A big drop in activity was observed at temperature higher than its optimum, that only 24% relative activity remained at 50°C.

The mentioned activity suggested that for prolong hydrolysis of noncooked cassava under conditions expected as unfavorable for contaminants (low pH and high temperature), strain N37 would offer better choice.

Temperature stability: To determine for temperature stability, the crude extract was incubated at various temperatures for 48 hours. Determination of raw starch digesting activity was performed under its optimum pH and temperature. It was shown in Table 7 and Fig. 11 that enzyme of strain N37 was very stable at temperature ranging from 4-45°C, whereas the enzyme of strain 102R was stable at temperature from 4-40°C (Table 8, Fig. 12). The results obtained thus agree to their optimum temperatures.

pH stability: In determination of pH stability of the enzyme, crude extract was mixed with 0.1 M citrate buffer of various pH and held at 4°C for 48 hours. Determination of raw starch digesting activity was performed after the pH was readjusted to its optimum pH (4.5) with citric acid or sodium citrate. It was shown in Table 9, and Fig. 13, that enzymes of strain N37 was

Table 5. Effect of temperature on raw starch digesting activity of crude enzyme extract of *Rhizopus* sp. N_{37}

Temperature °C	glucose liberated (mg %)	Relative activity
35	2100	85.1
40	2398	97.0
45	2467	100.0
50	2271	92.1

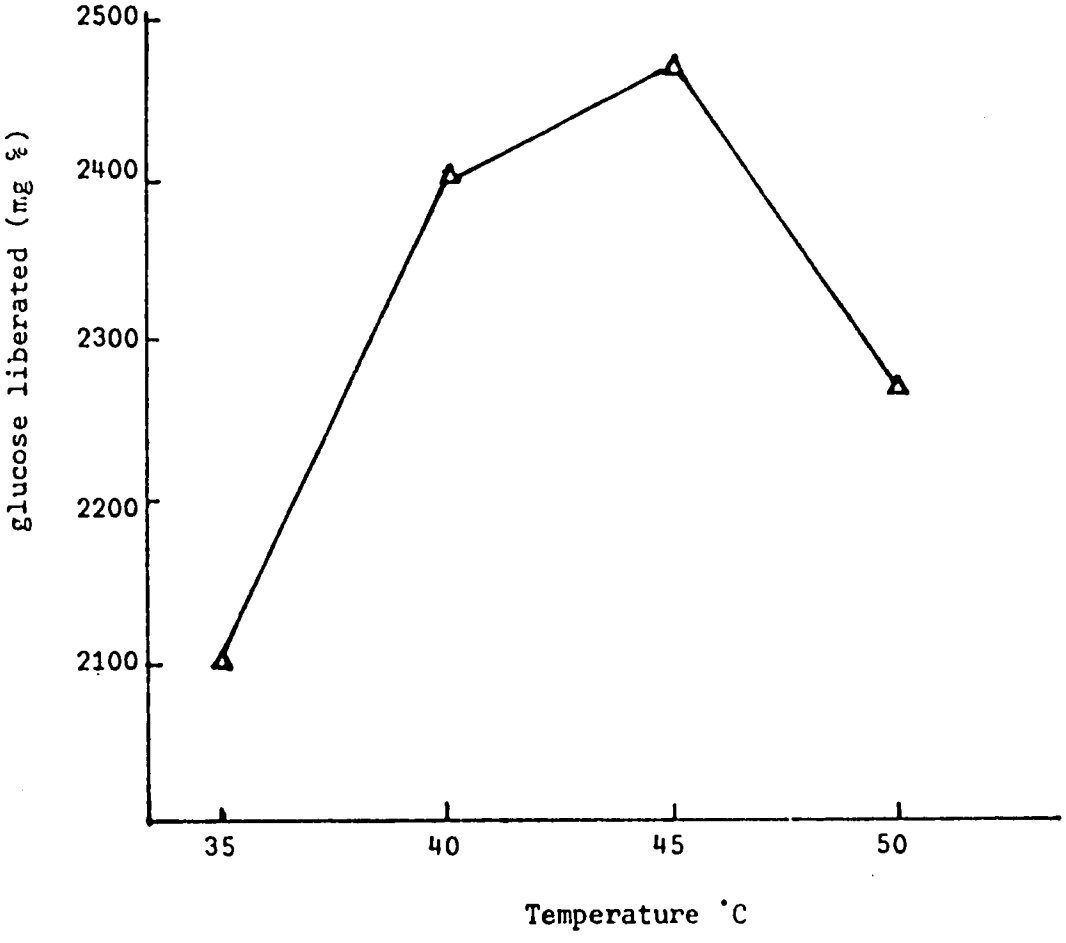


Fig 9. Effect of temperature on raw starch digesting activity of crude enzyme of Rhizopus sp. N_{37}

Table 6. Effect of temperature on raw starch digesting activity of the crude enzyme extract of *Rhizopus* sp 102 R

Temperature °C	glucose liberated (mg %)	Relative activity
35	1995	93.9
40	2125	100.0
45	1824	85.8
50	510	24.0

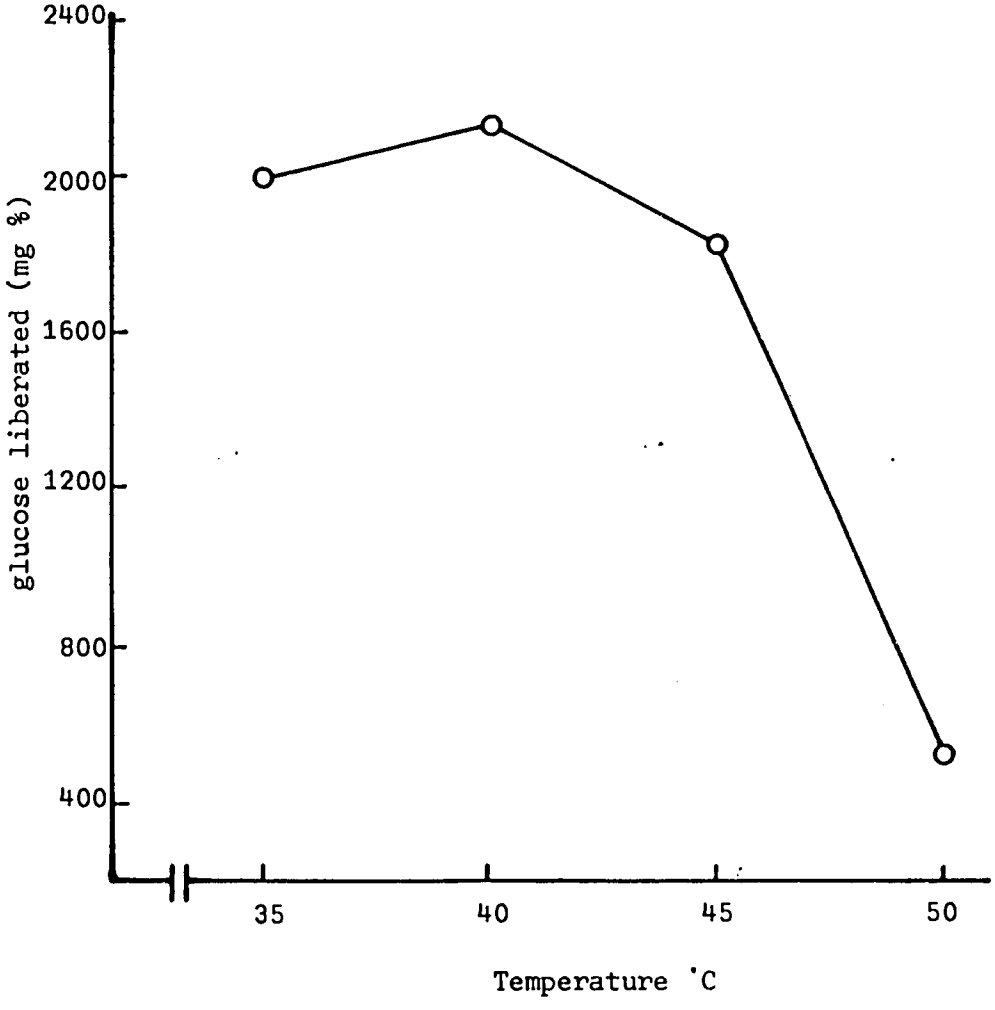


Fig 10. Effect of temperature on raw starch digesting activity of crude enzyme extract of Rhizopus sp. 102 R

Table 7. Effect of temperature on stability of raw starch digesting enzymes of Rhizopus sp. N_{37}

Temperature[1/] °C	glucose liberated (mg %)	Relative activity
4	1991	98.5
27	2005	99.3
40	2019	100.0
45	1948	96.5

1/ : the enzyme was incubated at each temperature for 48 hours.

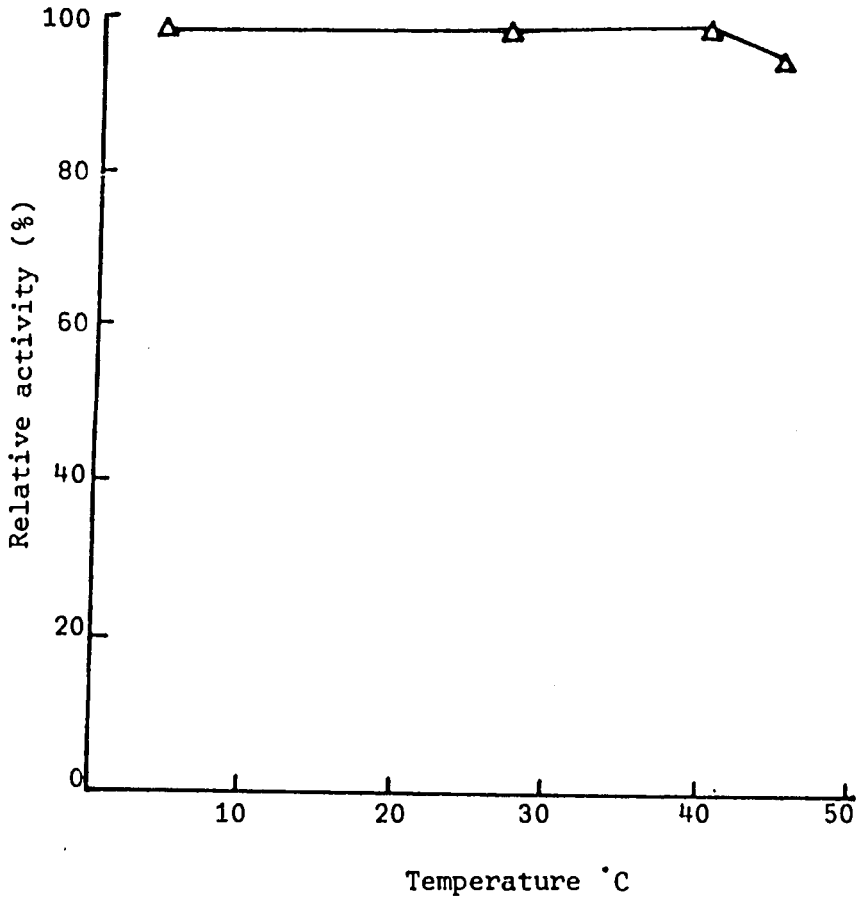


Fig 11. Effect of temperature on stability of raw starch digesting enzymes of Rhizopus sp. N_{37}

Table 8. Effect of temperature on stability of raw starch digesting enzymes of Rhizopus sp. 102 R

Temperature[1/] °C	glucose liberated (mg %)	Relative activity
4	1734	100
30	1690	97.5
35	1557	89.8
40	1657	95.6
45	1358	78.3
50	109	6.3
55	51	2.9

1/ : the enzyme was incubated at each temperature for 48 hours.

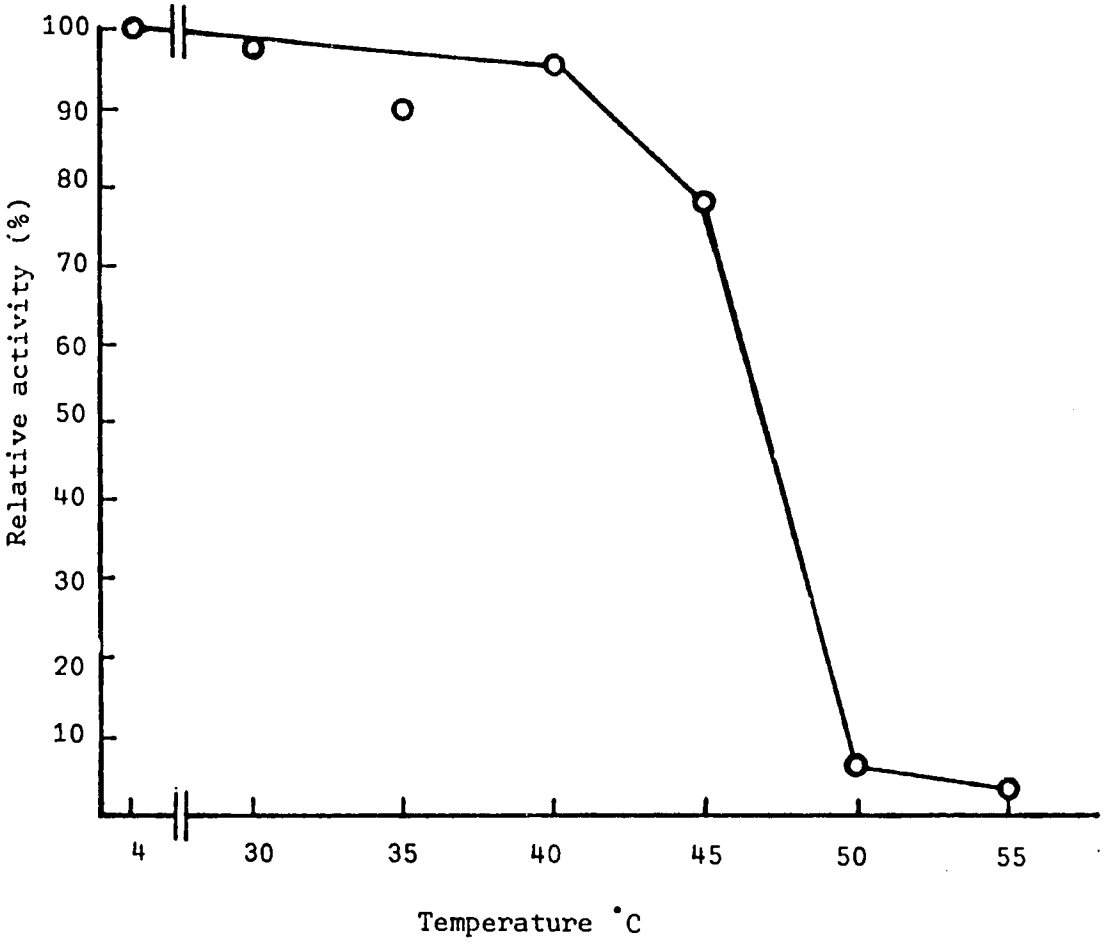


Fig 12. Effect of temperature on stability of raw starch digesting enzymes of Rhizopus sp. 102 R

Table 9. Effect of pH on stability of raw starch digesting enzymes of *Rhizopus* sp. N_{37}

pH	Hydrolysis product glucose (mg %)	Relative activity
3.5	440.23	18.45
4.0	1534.22	64.31
4.5	1949.19	81.69
5.0	2386.01	100
5.5	2062.22	86.42

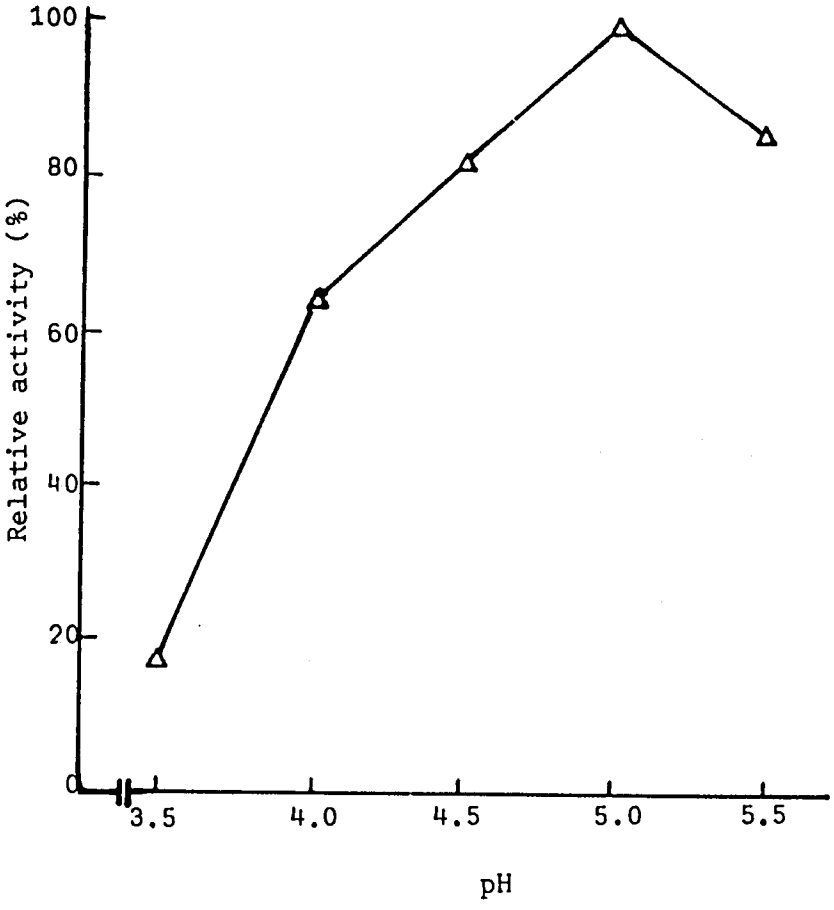


Fig 13. Effect of pH on stability of raw starch digesting enzymes of Rhizopus sp. N_{37}

Table 10. Effect of pH on stability of raw starch digesting enzymes of *Rhizopus* sp. 102 R

pH	glucose liberated (mg %)	Relative activity
3.5	947.34	49.52
4.0	1305.10	68.22
4.5	1648.20	86.16
5.0	1913.75	100
5.5	1856.90	97.07

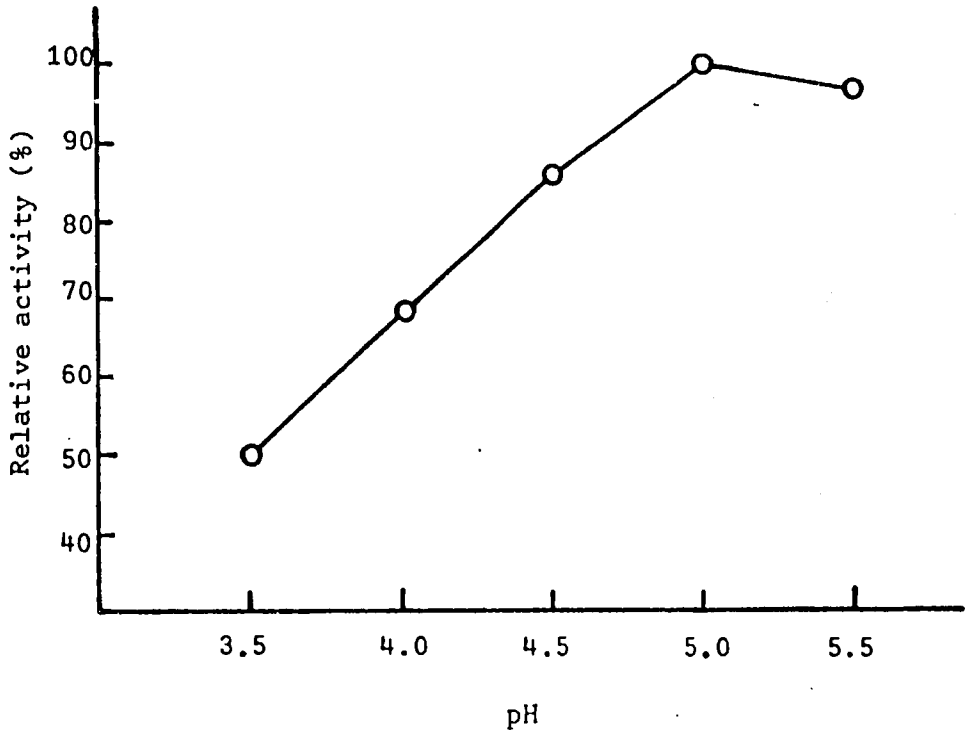


Fig 14. Effect of pH on stability of raw starch digesting enzymes of Rhizopus sp. 102 R

stable only at pH 5.0. The enzyme lost 19 and 14% activity upon storage at pH 4.5 and 5.5, respectively. It was quite clear that the enzyme of *Rhizopus* 102R was stable at pH ranging from 5.0-5.5 (Table 10, Fig. 14).

Alcohol Fermentation from Cassava Hydrolysate

Kinetics of Ethanol Fermentation by Selected Yeast Strains

Introduction

From the final screening in previous progress report, yeast strains Sc.90 and AM.12 showed higher ethanol fermentation in terms of both efficiency and rate. Therefore, these two strains were then studied in detail for their kinetics in a 2.5 L fermentor. Historically, there was no doubt about the performances of them in alcohol fermentation. The main purpose of this experiment was to confirm their ability in converting starch hydrolysate into ethanol. However, it should be kept in mind that starch hydrolysate used in the study was prepared from hydrolysation of cassava chip by commercial enzyme preparation, namely NOVO BAN (bacterial α-amylase) and SAN (Spiritamylase). Depolymerization of starch molecules to glucose was almost completed prior to carry out alcohol fermentation. Moreover, the medium could be sterilized to ensure maximum ethanol yield. Hence, fermentation was carried out in almost ideal condition for yeast growth. In contrast, the expected hydrolysation of starch by koji prepared from the project would be rather slow and yeast fermentation probably has to short before completion of starch digestion. Bacterial contamination would be quite serious, since, the mash could not be heat sterilized. Other means of controlling bacterial population may have to be determined and

probably selective bacteriostatic agent, like antibiotics might be appropriate. However, the study will be conducted later on.

Materials and Methods

1. Cassava hydrolysate was prepared as previously described.

2. Fermentation was carried out in a 2 liter-fermenter, EYELA, Model M-100, Tokyo Rikakikai, with the following condition:

- Working volume 1.5 liter
- Initial pH 4.5
- Temperature controlled at 37°C
- Agitation 150 rpm
- Inoculum size 3%
- Sampling at 0, 4, 8, 12, 16, 20, 24, 30, 36, 42 and 48 hrs.

3. Determination of fermentation parameters:

- Glucose by HPLC
- Ethanol by Gas Chromatograph
- Viable cell count by methylene blue staining and microscopic direct count.

Results and Discussion

Table 11 showed time course of ethanol fermentation by selected yeast strain, namely Sc.90 and AM-12. Strain Sc.90 which is a non-floculating yeast and being used in distilleries in Thailand grew quite well in the starch hydrolysate medium. However, as compared to molasses medium which is currently used in industries, the rate was rather slow. Generally, this strain could completely convert sugar at the concentration of 16-20% to ethanol within 30 hours with residual sugars (non-fermentable) about 2-3%. In starch hydrolysate medium, ethanol concentration reached about 7% after 30 hours and increased slightly until 48 hours. However, glucose concentration decreased to less than 2% after 24 hours, and remained at this level to the end of fermentation. On the other hand, total viable cells was slightly lower than usual. The maximum cell number was around $2x10^7$ cells/ml while in the previous experiment cell concentration reached $9.98x10^7$ cells/ml. This was probably due to lack of certain nutrients such as N-source or growth factors and O_2. In shaker flask, yeast obtained considerable amount of oxygen which would support cell growth, especially at the early phase of growth. In fermenter, availability of oxygen was limited at the beginning which would reduce growth rate.

Strain AM-12 which was a hybrid of industrial yeast strains yielded final ethanol concentration similar to Sc.90. However, alcohol production rate was rather lower. This was

Table 11 Time course of ethanol fermentation from cassava hydrolysate by selected yeast strains in 2-liter-fermenter at 37°C

Time hrs.	Sc.90			AM-12		
	Glucose %	Ethanol %	Log no. of viable cell	Glucose %	Ethanol %	Log no. of viable cell
0	16.82	0.19	6.13	17.06	0.21	6.18
4	16.03	0.65	6.48	16.95	0.33	6.25
8	14.38	1.61	6.75	16.04	0.54	6.50
12	9.27	3.16	7.04	13.61	1.38	6.76
16	5.01	5.17	7.23	10.48	2.49	7.13
20	2.96	6.30	7.31	8.89	4.08	7.21
24	1.90	6.68	7.38	6.23	4.90	7.38
30	1.55	6.97	7.26	3.20	6.00	7.44
36	1.73	7.12	7.21	2.77	6.52	7.49
42	1.40	7.29	7.22	2.33	7.15	7.45
48	1.38	7.44	7.05	1.92	7.64	7.55

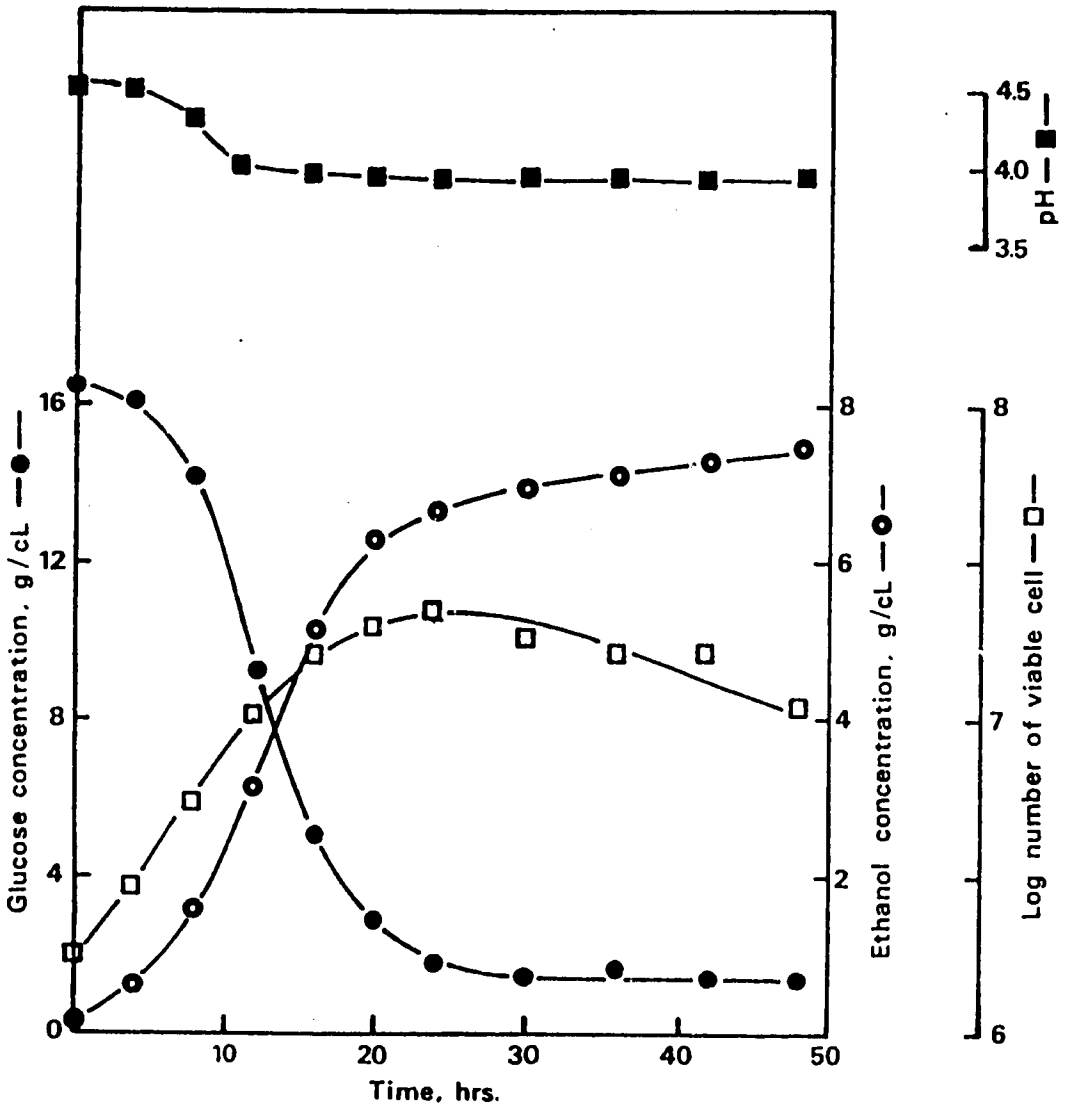


Fig.15 Time course of ethanol fermentation from cassava hydrolysate by *Sc. cerevisiae*, Sc90 in 2-liter fermenter at 37°C.

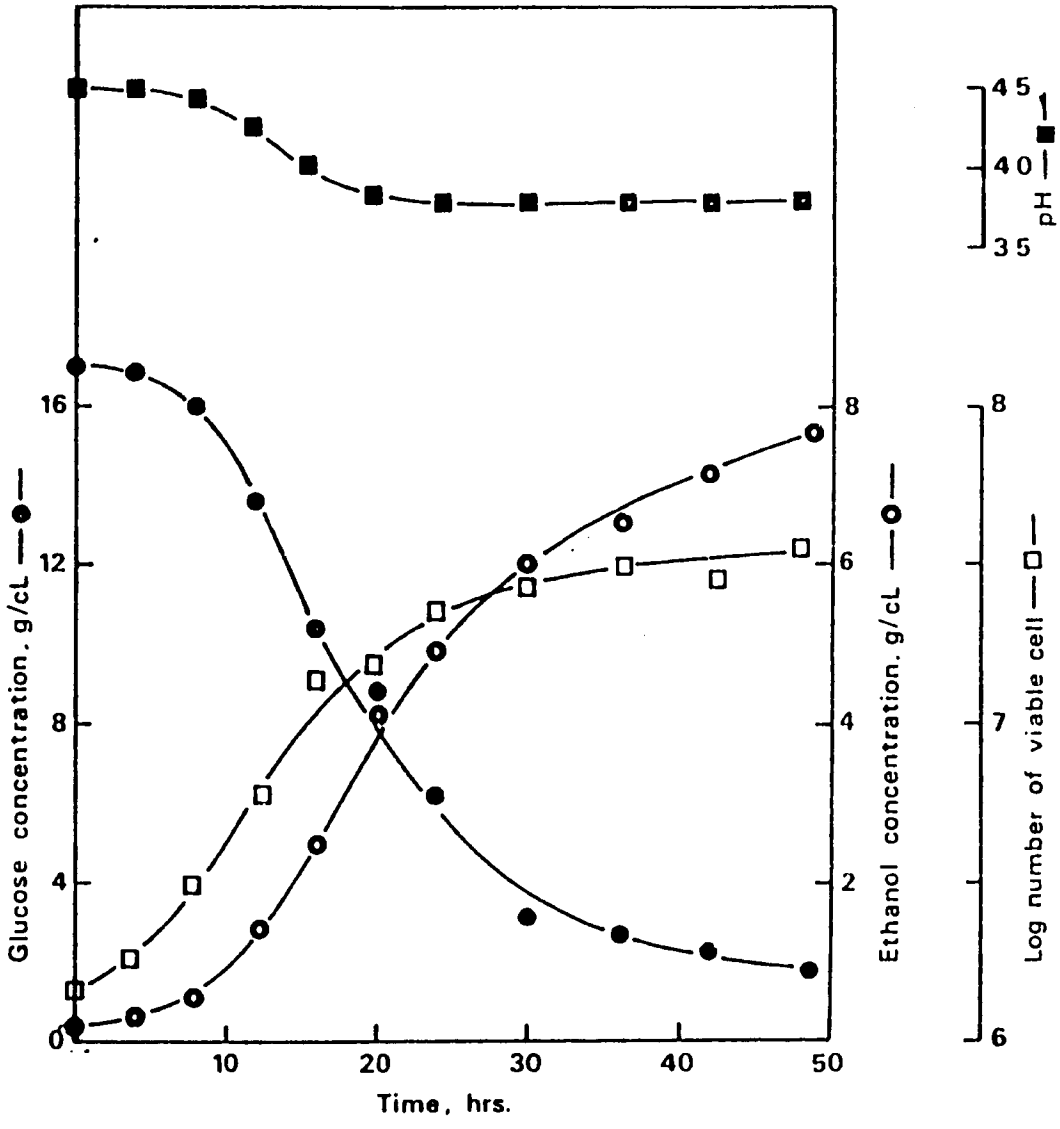


Fig.16 Time course of ethanol fermentation from cassava hydrolysate by *Sc. cerevisiae*, AM 12 in 2-liter fermenter at 37°C.

probably due to nature of floculating yeast. Glucose consumption rate was also lower as compared to Sc.90 where sugar concentration was at the level of 2% after 42 hours.

Conclusions and Remarks

1. Information obtained through the study on optimization of culture conditions for maximal enzyme production together with that optimal for the enzyme activities, provided a conclusive decision that except the pH stability, *Rhizopus* strain N37 is more competitive over strain 102R in term of practical application. Advantages of strain N37 was summarized as followed:

- The enzyme could be produced at lower pH, lower moisture content and higher temperature which would make it competitive to the contaminants during cultivation. Moreover, regarding on its thermotolerance, design of koji chamber would not need strictly control of temperature.

- The enzyme is active at a broader range of pH and temperatures.

- The enzyme is stable at broader temperature, which would make it more practical to work with, and easier in formulation.

2. From the kinetics of these two selected yeast strains, it seemed that Sc.90 was superior in terms of ethanol production and glucose consumption rates under the condition that starch was completely hydrolysed.

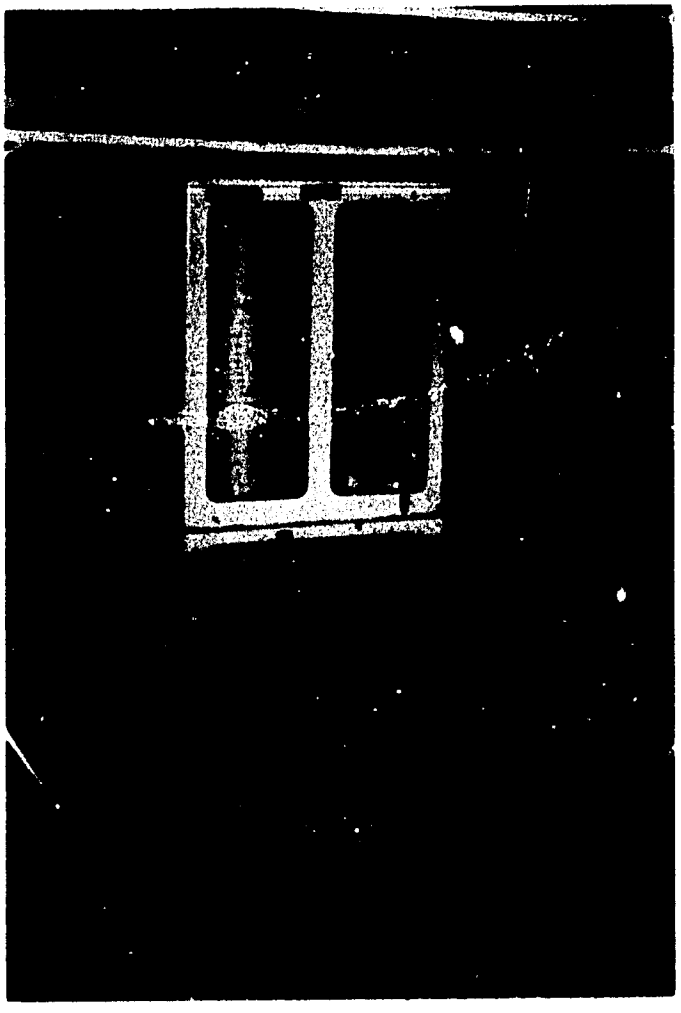
Work Plan for the Next Period

1. Study on raw starch hydrolysis time course using enzyme of *Aspergillus* J8 and *Rhizopus* 102R.

2. Determination for enzyme substrate proportion for complete hydrolysis.

3. Enzyme purification and characterization of the fractions which play important roles on raw starch hydrolysis.

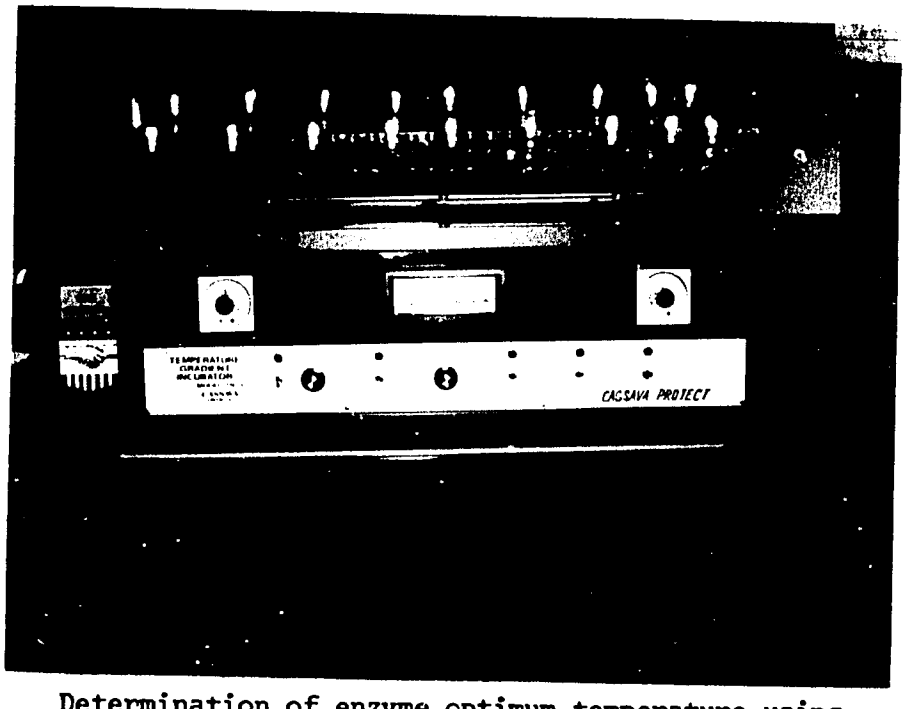
4. If alcohol fermentation has to be comed out simultaneously with hydrolysation, other two yeast strains, i.e. M-30 and 191 may be appropriate. Hence, the next experiments would concerned about simultaneous fermentations by all four yeast strains.



Wheat bran koji of Aspergillus sp in small Scale koji chamber

Analysis of raw starch hydrolysate using HPLC

Koji preparation in plastic bags



Determination of enzyme optimum temperature using temperature gradient incubator