https://doi.org/10.29321/MAJ.10.A04312

Enzyme (Diastase) of Aspergillus oryzae and its Application to Malt Extract Production*

by

S. VARADARAJAN,
Chemistry Section, Agricultural Research Institute, Coimbatore

I. **Introduction:** The method employed in the Government Malt Factory for the production of malt extract was by germinating cholam (*Sorghum durra*). Since the malted cholam contains comparatively little of the enzyme amylase, only about 20 per cent of the starch contained in the cholam grain is recovered as sugar (maltose). This tells on the cost of production of malt extract, besides the cost involved in the malting process where controlled conditions of temperature, humidity, constant attention etc., are necessary, for which special equipments in the form of air-conditioned couching chambers have to be maintained.

It has long been known that the fungus *Aspergillus oryzae* contains an enzyme of high diastatic power and, therefore, investigations on the enzymatic studies on *Aspergillus oryzae* were carried out to see its applicability to the production of malt extract. Because of its high saccharifying power (nearly four times that of malted cholam) it was hoped, that if a method was developed for the utilization of fungal diastase, the cost of production could be reduced considerably.

II. **Review of Literature:** The fungus was originally known as *Eurotium oryzae*, first being identified by Ahlberg in 1879, and later, Cohn renamed it as *Aspergillus oryzae*. O. Kellner and his pupils investigated the invertase, amylase and maltase content of it. Takamine, (1914) using wheat bran as culture medium, grew the fungus and gave the name "Taka-Koji" to it and used it as an amylo-clastic agent in fermentation industries. The chief advantage of using wheat bran instead of malt is that the cost of malt is subject to wide fluctuation according to the crop conditions. And secondly, the transformation of bran into "Taka-Koji" requires only about 48 to 72 hours while malting needs 3 or 4 times that period. Underkofler (1939) revived the idea of replacing malt with mold-bran in which the fungus elaborates the enzyme in the alcoholic fermentation of grains. Underkofler et al (1946) reported extensive commercial tests in which the yields of alcohol were as good or even better with mold-bran than with malt. The same authors (1947) reported successful commercial application of mold-bran for alcohol and syrup manufacture.

It is clear that the fungus is capable of producing diastase of very high efficiency; higher than that of barley diastase and has the added

*Paper awarded the Ramasastrulu Munagala Gold Medal on the College Day, 1954.

advantage of utilising various cheap by-products such as rice-bran, wheat-bran etc. for its growth for the production of the enzyme.

III. **Object of the Investigation:** It is known that cholam diastase is poor in comparison to barley diastase. It would certainly be advantageous to obtain diastase from the fungus and utilise it either alone or in combination with cholam diastase, whereby it might be possible to obtain greater yield of extract. Secondly, the cholam for malting should be of excellent quality. This is a factor to be considered when there is scarcity of grain with prevailing high cost. With fungus enzyme any kind of starchy flour can be used and, owing to the greater efficiency of the enzyme, the cost of production of malt extract could be reduced considerably.

IV. **Materials and Methods:** Preliminary experiments were conducted under the following heads:

(a) Selection of efficient strain of *Aspergillus oryzae* in regard to its diastatic activity.

(b) Selection of a suitable media for maintenance of the fungus.

(c) Comparing the efficiency of the two diastases, namely, cholam and fungal, after standardising the method of assay.

(d) Selecting the most suitable substrate for growing the fungus for the production of diastase.

(e) Working out the optimum conditions such as temperature, moisture, etc., for the growth of the fungus for best results.

(f) Developing a method for utilising the fungal diastase for production of malt extract.

(a) *Selection of efficient strain of fungus:* Strains possessing high diastatic activity were obtained from different places as shown in Table I below and their comparative efficiency tested.

TABLE I

No.	Details of isolate	Obtained from	Diastatic power, Lintner Units
1.	N. C. T. C. 558	Bangalore	93·63
2.	,, 598	England	87·50
3.	,, 965	England	73·48
4.	A. M. I. 16,266	U. S. A.	37·00
5.	,, 17,299	U. S. A.	53·04
6.	N. C. T. C. 595	New Delhi	123·00
7.	Isolated locally	Coimbatore	85·43

From the above test it is seen that N.C.T.C. 558 and 595 are the best and these were used in further experiments.

(b) *Selection of best media for maintenance of the fungus:* To maintain the fungus for work without deterioration of its activity a suitable medium was necessary. Standard media such as Brown's, Steinbergh's and Czapek's were tried for selecting a suitable one for the purpose. However these media were not suitable for the purpose contemplated. Therefore, a modified medium was prepared consisting of malt-extract, peptone and yeast water in addition to nitrogen, phosphate, sulphate and other mineral salts in traces.

(c) *Comparing the diastatic activity of the fungus.* (i) *Standardisation of assay technique:* The diastatic power of the fungus was estimated by three different methods, viz. (1) the method prescribed by A.O.A.C. (1935); (2) Ling's modification of Lunge and Keane's (1914) method and (3) by the method developed by Gore and Steele (1935).

Finally the A.O.A.C. method was adopted, after making certain modifications to suit the local condition, in all the experiments.

Assay in Brief: (i) *Cultivation of the fungus:* Wheat bran obtained locally from the bazaar was cleaned, dried and stored in kerosene tins. Samples of ten gram quantities were weighed out, 10 ml. of water added, sterilized, inoculated with five days old culture of the fungus grown on malt agar and incubated at 28°C for obtaining best growth in the bran. On the fourth day the diastatic activity was estimated as below.

(ii) *Extraction of the enzyme for assay:* The mold-bran (as it could be designated now) was spread on glass plate, samples of 5 gm. taken in a glass mortar, a few grams of washed sand added to it and with a small quantity of water was ground to a paste, transferred to a beaker and made up to 100 ml. to extract the enzyme. The solution was stirred frequently to disperse the enzyme uniformly in the liquid.

(iii) *Estimation of diastatic activity:* To 100 cc. of 2% starch solution kept at 55°C for 30 minutes with the addition of 5 ml. of sodium acetate buffer solution to maintain the pH at 4·8 (considered optimum for the enzyme), 3·ml. of the extracted enzyme solution were added. The flask was kept exactly for 1 hour when the enzyme action was arrested by the addition of 10 ml. of N/10 KOH solution.

The amount of maltose released by the action of the enzyme was assessed by titrating against 5 ml. of Fehling's solution and the diastatic power in Lintner units expressed by using the formula:

$\frac{1,000}{xy}$ where, x is the number of ml. of diastase solution added and y the number of ml. of the hydrolysed starch solution required to reduce 5 ml. of Fehling's solution.

V. **Results:** (i) *Comparative values of diastatic power of cholam and fungal diastases:* To test the efficacy of the fungal diastase for the production of extract it was compared with the enzyme of malted cholam.

Green cholam malt prepared in the Government Malt Factory at 65°F for 72 hours was used in the test. The values are given below:

TABLE II

	Substance	*Diastatic power in Lintner units at different temperatures*		
		28°C	40°C	55°C
1.	In Czapek's solution (mycelium)	Trace	Trace	Trace
2.	,, Liquid portion	2·01	3·23	3·77
3.	Grown on waste malt	16·55	30·54	33·71
4.	Green malt — (cholam)	14·22	26·69	34·36

The diastatic power of the fungus grown on waste malt was not greater than that of cholam malt. Other workers have reported very high diastatic power equivalent to barley malt and, hence, the reason for the low value was investigated into.

(ii) *Diastatic power of the fungus grown on rice and wheat brans with different sources of nitrogen.* The diastatic power of the fungus grown under controlled conditions on wheat bran, waste malt and rice bran with different sources of nitrogen was assessed and the results are given below:

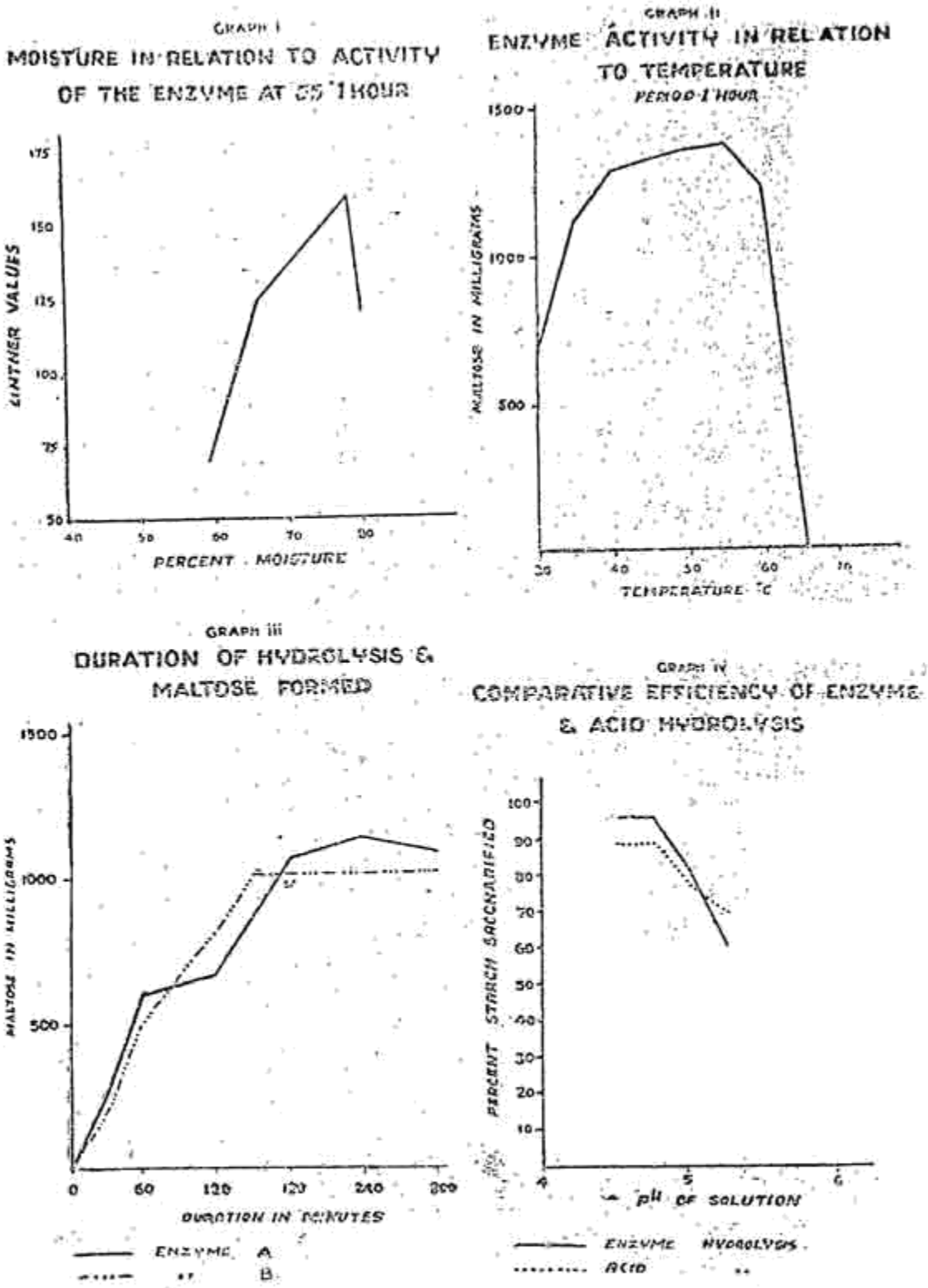
TABLE III

	Substance	Diastatic power at 55°C 60 minutes Lintner Units
1.	Waste malt 10 gm. + 0·25 gm. Ammonium sulphate	33·71
2.	,, + 0·25 gm. Ammonium phosphate	33·71
3.	,, + 1 gm. groundnut cake	37·77
4.	,, 5 gm. + 0·5 gm. groundnut cake	51·45
5.	,, 2·5 gm. + 0·25 gm. groundnut cake	101·07
6.	Rice bran 10 gm. + 1 gm. groundnut cake	82·47
7.	,, 5 gm. + 0·5 gm. groundnut cake	56·14
8.	Wheat bran 10 gm. + 0·25 gm. Ammonium phosphate	114·18
9.	,, ,, + 1 gm. groundnut cake	140·13
10.	,, 5 gm. + 0·5 gm. groundnut cake	73·64
11.	,, 5 gm. + Rice bran 5 gm.	125·54

The following conclusions are drawn from the data obtained.

(1) The diastatic power of the enzyme produced by the *Aspergillus oryzae*, is far superior to that of cholam malt. The former is three to four times greater than that of the latter.

(2) Of all the substrates tried for the growth of *Aspergillus oryzae*, wheat bran has proved itself to be the best consistently in point of producing diastase of very high power.



(3) The fungus makes good growth on waste malt. But, since this substance is practically three-fourths starch, the fungus utilises only a fraction of it and the rest is wasted.

(4) Rice bran as a substrate is not as good as wheat-bran Addition of wheat-bran in equal proportion increases its value.

(iii) *Utilization of other raw materials for the production of diastase:* Several previous investigators including Bindal and Sreenivasaya

(1944) have used wheat-bran, oil-cakes, cornmeal, crushed soya-bean meal etc. either as exclusive substrates or as supplements to the basal medium. Of the materials investigated wheat-bran has been found to be the substrate *par excellence* for the growth of *Aspergillus oryzae*.

For the production of enzyme a medium should be such that it will promote vegetative growth in abundance with a high diastatic content. The medium should not stimulate spore formation. Of the raw materials tried, besides wheat and rice-brans, those that have no feeding value but only valuable as fuel such as Ragi bhusa, dhall husk, groundnut shells etc. may be mentioned. They were used after pounding them to fine powder. But they do not compare favourably with wheat bran.

TABLE IV

Substrate	Lintner units
	55° – 60'
Groundnut bhusa	21·2
Ragi bhusa	15·8
Dhall husk	25·7
Ragi bhusa & groundnut cake	28·4
Dhall husk and groundnut cake	29·5
Wheat bran	82·6
Wheat bran & groundnut cake	112·4

(iv) (a) *Enzyme activity in relation to moisture content of the substrate:* To arrive at the optimum level of moisture necessary to moisten the bran for the best activity of the fungus; to 10 gm. portions of wheat bran in conical flasks water was added in varying amounts, sterilised and after the flasks were inoculated with fungal spores, they were incubated for 7 days at 28°C. The enzyme was extracted at the end of the period and its activity measured.

TABLE V

Enzyme activity in relation to moisture content of substrate

Moisture %	Lintner units 30 min. 55°C	Lintner units 60 min. 55°C
58	21·43	70·70
60	24·08	82·50
65	44·85	125·54
70	56·42	140·24
75	58·75	150·07
78	62·41	159·50
80	60·80	122·60

The activity of the enzyme is represented in graph I. The activity increases upto 78% moisture and drops at higher levels. Therefore it is clear that the bran should be moistened to 75 to 78 per cent level for the best elaboration of the diastase.

(b) *Enzyme activity in relation to time : (temperature constant.)* To find out how long the hydrolysis should be continued at a constant temperature for the maximum activity of the enzyme so that the greatest amount of starch could be saccharified.

A set of flasks containing 100 ml. of 2% starch solution was kept at 55°C. in a thermostat and the pH was adjusted to 4·5 by adding 5 ml. of sodium acetate buffer. The enzyme action was arrested at the specified period by adding 10 ml. of N/10 KOH solution and the maltose formed was estimated by the usual method. The results are given in Table below.

TABLE VI

Duration of hydrolysis and maltose formed at 55°C

Duration in minutes	*Maltose formed in mgm.*	
	Enzyme from sample A	Enzyme from sample B
30	275	245
60	600	519
120	653	818
180	1080	1013
240	1141	1019
300	1120	1025

From the data it is clear that the rate of saccharification is found to increase with the increase in time. The enzyme activity was practically same after three hours. The rate and course of action were almost identical when two different strains were used (vide graph iii).

(c) *Enzyme activity in relation to temperature:* Experiments with a view to find out the optimum temperature at which maximum of enzyme action takes place was carried out as below.

A set of flasks containing 100 ml. of 2% starch solution was adjusted to pH 4·8 and kept at temperatures varying from 30 to 70°C. for 1 hour and the maltose formed was estimated.

TABLE VII

Enzyme Activity in Relation to Temperature

(Period—1 hour)

Temperature °C	Lintner units 55°C 60 min	Maltose mgm.
30	76·2	700
35	125 9	1,157
40	142·2	1,306
45	146·9	1,350
50	149·4	1,373
55	152·0	1,396
60	137·7	1,266
65	trace	98
70	nil	nil

The activity of the enzyme increases steeply at first and steadily after 35°C. with the increase in temperature upto 55°C. and then declines rapidly. (vide graph ii). The maximum activity is thus seen to be around 55°C.

(v) *The effect of hydrogen-ion concentration of the substrate and enzyme activity.* It is well known that the optimum activity of the enzyme depends on the hydrogen-ion concentration of the solution with which it reacts. In order to select the level of pH of the solution for optimum activity of the enzyme an experiment was carried out and the data are given below. Side by side the acid hydrolysis of starch is given for comparing the efficiency of the enzyme with the acid hydrolysis.

TABLE VIII

pH of starch solution	Starch present	*Enzyme hydrolysis*			*Acid hydrolysis*		
		(a)	(b)	(c)	(a)	(b)	(c)
5·5 enzyme	6·741	4·574	4·117	61·07	5·208	4·687	69·54
5·0 ,,	,,	6·175	5·558	82·45	5·883	5·294	78·36
4·5 ,,	,,	7·265	6·539	96·98	6·822	6·001	88·98
4·0 ,,	,,	7·265	6·539	96·98	6·822	6·001	88 98
4·0 (control)	,,	Nil	Nil	Nil	—	—	—

(a) Sugar as maltose—gm.; (b) Starch equivalent—gm.
(c) Starch saccharified—%.

The maximum activity of the enzyme is between pH 4·0 and 4·5. At these pH, by the enzyme hydrolysis the saccharification is 96·98% while, by the acid hydrolysis it is only 88·98%. Thus it is seen that the activity of the enzyme is greater than that of the acid between pH 4 and 4·5. (Graph iv).

(vi) *Activators in relation to enzyme activity.* Some organic and inorganic substances such as amyl alcohol, ethyl alcohol, glycerol and iron, copper, manganese and zinc as catalysts were tried in small amounts as activators to speed up and enhance the activity of the enzyme.

No marked activity was noticed due to addition of the activators though there were differences among the several reagents tried in regard to their influence.

(vii) *The duration of fungal growth in relation to its activity:* To find out when the fungus attains its maximum activity in regard to the enzyme, the two strains of the fungus were cultivated on malt-agar and their activity measured daily from the 3rd to 6th day. The results are given below:

TABLE IX

Isolate		3rd day	4th day	5th day	6th day
N. C. T. C. 558	A	105·5	111·4	98·6	86·2
	B	112·57	114·5	75·6	85·4
N. C. T. C. 595	A	118·4	163·9	159·0	122·6
	B	138·9	161 5	150·1	119·5

(Values in Lintner units)

The diastatic power between third and fourth day is maximum and falls markedly later in both the cases.

VI. **Studies on the Utilisation of the Enzyme:** *Utilisation of mold-bran for the preparation of malt extract.* The saccharifying capacity of the mold-bran on the starch present in cholam, was examined as below

Preparation of Mold Bran: The mold-bran was prepared by cultivating the strain selected for its best growth and high enzyme activity, namely isolate No. 595, on sterilised moistened wheat-bran to which 10% of groundnut cake was added. The fungus was allowed to grow in a dark place at room temperature for three days by which time the maximum of mycelial growth developed. (Used on the fourth day).

A sample of cholam, known as "Patcha jonna", was ground to a coarse flour and used for the experiments reported below.

(1) *The quantity of mold-bran for maximum saccharification of the starch present in cholam:* With the idea of finding out the quantity of mold-bran required in relation to the amount of cholam consistent with the maximum recovery of sugar the following experiment was carried out.

To 10 gm. lots of cholam flour 100 ml. of water were added and raised to a boil to mash the flour, kept at that temperature for 2 minutes, cooled to 55°C and mold bran in varying quantities added and the action of the enzyme was allowed to proceed for one hour, boiled again for a few minutes to destroy the enzyme, filtered and sugar estimated in the filtrate. The results are given below:

TABLE X

Quantity of mold-bran	% Starch saccharified
1 gram	54
2 ,,	54
3 ,,	67
4 ,,	67
5 ,,	71
6 ,,	71
7 ,,	71
8 ,,	72
9 ,,	72

The best result obtained for the least quantity of bran was for 5 gm. of mold-bran.

The next step was to adjust the pH of the mash for, though the percentage of saccharification of the added starch was very high, it fell

short of the ideal which is 100%. Hence the pH was adjusted and the results are as below :

Mash acidified to pH	Per cent starch saccharified
5·5	61
5·0	83
4·5	97
4·0	97
4·0 (Control — No enzyme)	Nil

At pH 4·0 to 4·5 maximum hydrolysis of starch took place. Hydrolysis due to acidification of mash for pH adjustment was nil.

(b) *Gelatinising the starch present in cholam and saccharification :* Gelatinising the starch by boiling the cholam flour mash for a short while gave nearly three and a half times increased saccharification.

	Percent starch saccharified
Un-cooked	24·73
Gelatinised (cooked)	86·02

VII. **Practical Application of the Enzyme for Production of Malt Extract :** Preparation of malt extract with mold-bran was attempted as given under.

(1) *Method :* One pound of cholam, ground to a coarse flour, was cooked in water to gelatinise the starch, the mash kept at 55°C, pH adjusted to 4·5 with N/10 sulphuric acid and half-a-pound of mold bran (3 days growth) added. The hydrolysis was continued for 3 hours. After hydrolysis the liquid portion was separated by filtration through a muslin cloth and enough lime water to neutralise the sulphuric acid and precipitate it as sulphate of lime, was added (litmus test). Then 2 gm. of activated carbon for every 100 ml. of filtrate was added and filtered through activated carbon. The clear fluid was concentrated to a thick syrup of specific gravity 1·40 by evaporation under reduced pressure. The consistency of the extract, colour, flavour etc., were all perfect in every respect but there was slight bitterness in taste.

(2) *Bitterness and its removal :* The cause of bitterness was traced to the wheat bran. Apparently it contains some bitter principles that is responsible for the taste. The following experiment was carried out with a view to removing the bitterness in the final extract.

(i) Wheat bran cooked in 5 times its weight of water, the water discarded and the bran washed several times.

(ii) Wheat bran shaken for 1 hour in a shaking machine with 5 times its weight of water, the water thrown out and the bran washed well in several changes of water.

(iii) Wheat bran soaked in 5 times its weight of water overnight, the water thrown out and the bran washed in several changes of water.

The wheat-brans receiving pre-treatment as stated above were used in separate lots as substrate for growing the fungus and used for saccharification.

It was noticed that the extract obtained from the above treatments were all free from bitterness. Hence the simplest of the treatments, namely, soaking the wheat-bran in water over-night, removing the water and washing the bran in several changes of water was adopted as the best method. But, wheat bran when washed did not support the best growth of the fungus. An experiment was carried out to remedy this defect as per plan below:

Treatments.

(a) Wheat bran not washed (control)
(b) ,, plus 10% groundnut cake
(c) ,, washed
(d) ,, washed plus 10% groundnut cake
(e) ,, washed plus 1% starch.
(f) ,, washed plus 1% groundnut cake plus 1% starch.

The diastatic power was determined in the usual way and the following conclusions arrived at —

(i) Washing the bran prior to inoculation considerably reduced the diastatic power. This was due, mostly, to the poor growth of the fungus on washed bran.

(ii) The poor growth in the washed bran is presumably due to the removal of starch from the bran, since, the addition of starch restored the activity of the fungus.

(iii) Probably the growth promoting factors if any that were lost by washing were available to the same extent in the added cholam flour to support best growth.

(3) *Final method evolved to produce malt-extract:* Laboratory scale preparation was attempted by finalising the method of malt extraction as outlined below: To the washed wheat bran 1% of cholam flour was added, the fungus cultivated and this bran was used for saccharification of cholam flour. The cleaned cholam was slightly roasted, ground into a coarse flour, water (five times that of cholam) added, mashed after boiling for a while at 55° C. and the mold-bran (3 days growth) added. After three hours of hydrolysis the liquid was filtered through activated carbon. To the filtrate, small quantity of sodium bicarbonate solution was added. The hydrolysate was made neutral by the addition of calcium hydroxide (litmus test) and filtered and evaporated under vacuo. The extract produced in this way had a transparent amber colour, sweet taste and pleasant aroma. With

the knowledge and experience gained by these preliminary studies, semi-pilot scale preparation of the extract was made from cholam flour successfully which had excellent keeping quality.

Summary and Conclusions: A method for utilising fungal enzyme (diastase) for the preparation of malt extract in place of germinated cholam, with a view to reducing the cost of production of malt extract, was developed, and it was found that the fungal diastase can very well replace cholam malt for the production of extract. The fungus is cultivated easily on cheap by-products like wheat bran and no laborious or time consuming process is involved in the method. The cost of production comes to about a third of the method that had been employed in the malt factory. The extract prepared was fine in all respects and kept well for a considerable time. The studies revealed that the fungal enzyme can, therefore, be utilised for the production of extracts on a large scale at a cheap cost.

Acknowledgments: The author acknowledges his great indebtedness to late P. D. Karunakar, M. sc. (Rutgers), the then Government Agricultural Chemist for his keen interest and guidance in these studies and to Sri M. Sanyasi Raju, M. sc. (Wisconsin), the Government Agricultural Chemist for facilities offered to prosecute the studies later. Thanks are also due to the Heads of various Institutes who supplied type cultures for the study. The author is also indebted to Dr. A. Mariakulandai, M. sc., Ph. D., (Washington) for his valuable criticism and helpful suggestion in the preparation of this paper.

BIBLIOGRAPHY

1. A. O. A. C. (1935) — 4th Edn: XIV, 46 – p. 158, Washington.
2. Beresford, H. and Christensen, L. M. (1941) — Idaho Agr. Expt. Sts. Bull 241.
3. Bindal, A. N. and Sreenivasaya M. (1944) — Sci. and Ind. Res., Vol. 3, No. 6, p. 245.
4. Gore, H. C. and Steele, H. K. (1935) — J. Ind. Eng. Chem. Anal. Ed. 6, No. 5, 324 – 326.
5. Hao, L. C., Fulmer, E. L. and Underkofler, L. A. (1943) — J. Ind. Eng. Chem. 35 : 814 – 818.
6. Hao, L. C. and Jump, J. A. (1945) — J. Ind. Eng. Chem. 37 : 521 – 525.
7. Lunge, G. and Keane, C. A. (1914) — Gurney & Jakson, London, Vol. III, part 2, p. 697 – 698.
8. Takamine, J. (1914) — Ind. Eng. Chem. Vol. VI, p. 824.
9. Underkofler, L. A., Fulmer, E. L. and Schone, L. (1939) — Ind. Eng. Chem. Vol. 21, No. 6, p. 734 – 738.
10. Underkofler, L. A., Severson, G. M. and Goering, K. J. (1946) — Ind. Eng. Chem. Vol. 36, No. 10, p. 980 – 85.
11. Underkofler, L. A., Severson, G. M., Goering, M. J. and Christensen, L. N. (1947) — Cereal Chem. XXIV, No. 1.