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Studies on Transportation of Wet Fish. I. Impact of Packaging on Cost

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The cost of transportation of wet fish in iced and frozen condition in different types of returnable and non-returnable containers was analysed. Transportation of wet fish under frozen condition is less economical compared to iced fish over short distances (below 1000 km) irrespective of the type of container used for transport. As no economic benefit can be derived from larger containers, it is recommended that the capacity of a container should be about 50 to 70 kg.

In India, about fifty to sixty thousand tons of fresh fish are transported annually by rail from the landing centres to major inland markets. Except a few refrigerated vans which transport about 5000 tons , almost all fish is transported in non-insulated parcel vans. Although the time of transport over the railways may vary between 24 and 70 hr, the total time elapsed from the point of landing to point of retailing may be as high as 80 hr. Short and medium distance transport is usually done by trucks or insulated road vans which cover a maximum distance of 600 km. in 20 hr. However, a recent report[1] showed that trucks are being used in long distance transport of fish which can cover a distance of 2000 km in 48 hr.

Ice is the only cooling medium used in transportation of fish. The quantity of ice used varies between 50 and 100 per cent of the weight of fish depending on the ambient condition, duration of journey and type of container. Reicing is done at intermediate points enroute whenever required. Type, capacity and cost of container used for fish transport varies from place to place. In general, bamboo basket lined with leaves, cane and creeper basket, plywood box (used tea chest) lined with jute cloth are extensively used for fish transport.

In the present study, the iced and frozen fish were transported from various points to Calcutta on experimental basis. The data of field experiments were analysed for finding out the differences in the average expenditure involved in transporting fish (both in iced and frozen state) in different containers.

Materials and Methods

Container: Size, capacity and materials of construction of different types of containers used in this study are given in Table 1.

Selection of procurement place: Different varieties of sea fish like *Dhoma* (*Scioena* sp.), mackerel (*Rastrelliger* sp.), Bombay duck (*Harpondon* sp.), pomfret (*Stromateus* sp.) and cat fish (*Siluridoe* sp.) were procured from Paradeep port, a major fish landing centre in the Eastern region of India (distance about 800 km. from Calcutta). Fish consignments took 34 hr to reach Calcutta market from the procurement place at Paradeep. Some quantity of similar variety of sea fish were also procured from Madras in Southern India (distance is about 1700 km from Calcutta). The fresh water varieties of fish like Rohu (*Labeo rohita*), Mrigal (*Cirrhina mrigala*) and catla (*Catla catla*) having an average weight of 200 g and length 6 to 8 in. were procured from Madras. Fish consignments took 62 hr to reach Calcutta market from the procurement place at Madras. All fish consignments were transported in non-refrigerated railway vans.

Packing: The capacity of each container varied from 50 to 100 kg. Fish and crushed lump ice (1:1 by weight) were packed in layers. Fish landed in the evening was immediately iced to bring down its temperature. Chilled fish is then packed in containers and despatched at night. The samples of fish were taken to laboratory for assessment of quality. Fish temperature was recorded immediately after arrival of consignments.

Freezing: The fish was frozen in a Model S.A. Tekamsha plate freezer. Fish received from different places was examined for their temperature, immediately iced, washed with chlorinated water and chilled prior to freezing. Fish were arranged in aluminium trays (28 cm × 18 cm × 5 cm) and interspaces filled up with water. The trays were then charged into the freezer. The temperature of the freezer was maintained at -30°C. An average time of 3½ hr was required for

TABLE 1. DETAILS OF CONTAINER USED IN COST ANALYSIS

Container No.	Description	Capacity (kg)	Weight (kg)	Cost (Rs.)
1.	Plywood box	50	5.00	7
	,,	75	10.00	10
	,,	100	15.00	15
2.	Plywood box with 400 gauge polyethylene film lining inside	50	5.25	10
	,,	75	10.50	15
	,,	100	16.00	20
3.	Plywood box lined inside with 1 cm thick expanded polystyrene in polyethylene bag[a]	50	5.50	9
	,,	75	11.00	12
	,,	100	16.50	15
4.	Moisture proof corrugated fibre board box with wood wool insulation in between two walls.	50	2.00	7
	,,	75	3.00	10
	,,	100	4.00	15

[a]Expanded polystyrene insulation can be used twenty times, which has been taken into consideration for calculation of cost.

complete freezing of fish. The blocks were then removed from the trays, packed in containers and despatched.

Measurement of fish temperature: The temperature of fish was measured by the procedure described by Graham[2] with a copper-constantan (0.05 cm dia.) thermocouple connected to a portable potentiometer.

Basis of cost analysis: In order to transport fish at acceptable quality level, the expenditure incurred consists of costs towards icing or freezing, cost on container and freight charges. It is assumed that all containers used in this study are freely available at all despatching centres at a fixed price. On the basis of available information, the freezing charge is taken as 50 paise per kilogram throughout the entire fishing season. The costing is based on freight charges applicable to perishable goods on Indian Railways during 1975-76. Equal amount of ice and fish (weight basis) were packed in all containers and the same quantity of ice (100 per cent of the weight of fish) was used in reicing of fish whenever required. It was noted that larger quantity of fish can be packed in a single container if it was on the frozen state (40 per cent more than the iced condition). All containers except the expanded polystyrene insulants are for single use and the above insulants can be used for twenty times. This however, requires extra care for collection, cleaning with suitable disinfectant and returning them to the despatching centres. The cost towards such operations was calculated as 20 paise per kilogram of fish and this is included during cost analysis for this particular container (container 2).

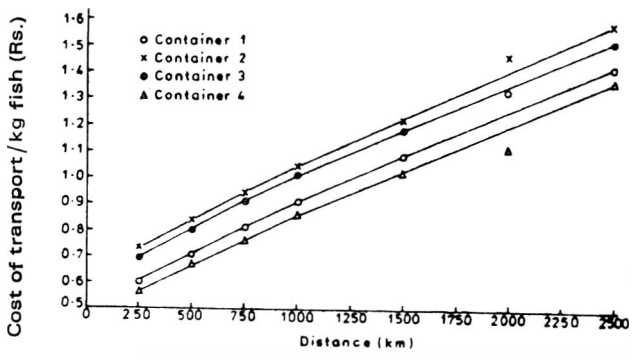

Fig. 1. Cost of transport of iced fish using different containers

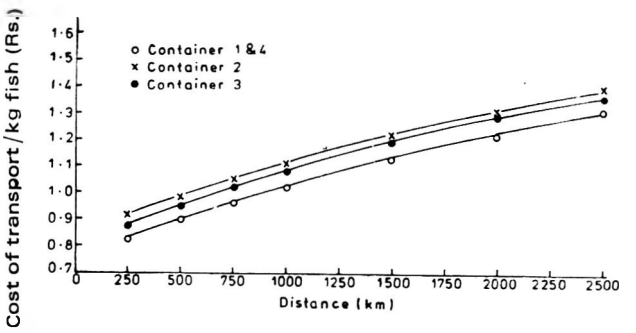

Fig. 2. Cost of transport of frozen fish using different containers

Results and Discussion

The cost of transport of one kilogram of iced fish in four different containers is shown in Fig 1 and that of frozen fish in Figure 2. Both the figures indicate the comparatively lower cost of transport using disposable fibreboard container (container 4). It is also evident that the transport of frozen fish is less economical compared to iced fish over short distances. As the distance between landing and consuming centre increases, the cost of transport of iced and frozen fish tend to come nearer. Freezing is needed only when distances are longer than 1700 km. This is because larger quantity of fish in frozen form can be transported in a single container directly thus avoiding reicing and related expenditure at intermediate points. The effect of gross weight of container on the cost of transportation of iced and frozen fish is negligible (difference is only 0.01 to 0.02 rupee per kilogram of fish). As no economic benefit can be derived from the increased capacity of container, the capacity should be limited to 50-70 kg. Moreover, smaller container can be handled with lesser effort. The above figures also indicate that fibreboard container can be used for transportation of both frozen and iced fish. Both for short and long distance transport, the cost of transportation per kilogram of iced fish in

fibreboard container is considrably lower than that of conventional plywood box and the cost is identical if fish is transported in frozen state

Acknowledgement

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Studies on Transportation of Wet Fish. II. Effect of Transport Environment on the Bacteriological Quality of Fish

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The bacteriological condition of vehicles and containers used in fish transport in India were investigated. Detection of large number of pathogenic organisms indicated the insanitary condition under which fish is transported in the country. This can be avoided using disposable fish container like fibre board box. Bacteriological condition of fish, both in iced (1:1) and in frozen state transported in such containers, were compared with those transported in reusable containers.

Marine fish are always contaminated by bacteria; the quality of fish is determined by the activities of these bacteria, apart from the autolytic and biochemical changes taking place in fish simultaneously. By intimate mixing with ice the lag phase of these bacteria can be extended from 4 to 5 hr to 5-6 days and thus the deterioration caused by bacterial activity can be minimised. In this contextit may be mentioned, that the nature of microflora of inland waters and fishes is not yet fully understood[1,2].

Apart from the marine microflora naturally present on fish, it is also contaminated with the bacteria of terrestrial origin as soon as it is dumped from the net on the deck of the fishing vessel. Some of these bacteria are important from the point of view of public health. Presence of these organisms beyond certain stipulated limit on fish may render it unfit for human consumption. The extent of contamination largely depends on the mode of handling, the care bestowed and the general sanitary conditions of the environment[3].

The bacterial load on fresh fish mixed with ice may alter as the fish is transported from one place to another depending on the time required for such transport and also on the bacteriological quality of ice employed. Data collected on all these aspects through actual field experiments are reported here.

Materials and Methods

Preparation of swab for bacteriological analysis of container and vehicle surfaces: Swabs were made of absorbent cotton (1.5 cm dia., 5 cm long) on a 15 cm glass rod, 2.5 mm dia. The swab, contained in a tube, was autoclaved at 121°C for 20 min. The area swabbed was 5 cm wide and 5 cm long using a tinplate swab guide which was sterilized by alcohol and flaming before use. The area was sampled by rotating the swab on it, firmly against the direction of wiping and then transferring it immediately into 100 ml of normal saline; the handle was then pulled free from the swab and discarded. The bottle containing the swab was shaken thoroughly. Decimal dilutions were prepared for bacteriological analysis.

Preparation of fish sample: About 10 g (surface area 6 sq. cm.) of fish was taken from dorsal side of the fish and blended with 100 ml of normal saline and 10 g of sterile sand. The clear supernatant was taken for serial dilution for bacteriological analysis.

Total bacterial count: The standard media[4] used consisted of the following ingredients: tryptone, 0.5; per cent beef extract, 0.3 per cent; sodium chloride, 0.5 per cent; glucose, 0.1 per cent; agar 1.5 per cent. The pH oft he medium was adjusted to 7.2 by sodium hydroxide solution and autoclaved at 15 psig for 15 min. After

serial dilution, samples were plated in triplicate and plates were incubated at 37°C for 48 hr. Visual colonies were counted.

Faecal Streptococcus: The KF agar medium[5] was used for this purpose. The pH of the medium was adjusted to 7-7.2 with sodium hydroxide solution. After addition of the indicator (Bromocresol purple), the medium was autoclaved at 15 psig for 15 min. One millilitre of 1 per cent sterile 1:3:5 triphenyl tetrazolium chloride solution was added to each 100 ml of sterile KF agar medium before pouring into plates. The tetrazolium solution was sterilized immediately before use by boiling it for 5 min. Plates were incubated at 37°C for 48 hr. The red and pink colonies developed were counted as faecal *Streptococci.*

Coliforms and E. coli: The Desoxycholate Agar medium was used for detection of coliforms and *E. coli.* Ten colonies were picked up randomly and inoculated into Eijkman Lactose medium in tubes containing Durham's tube and incubated at 45.5 ± 0.5°C. The production of gas is indicative of the presence of *E. coli* type I.

Staphylococcus count: The Chapman Stone medium was used for detection of *Staphylococcus.* After serial dilution, sample was spread on the surface of the preset medium using a bent glass rod and incubated at 37°C for 48 hr. With ten randomly selected yellow or orange colonies the following tests were performed: (*a*) fermentation of mannitol (*b*) gelatin liquefaction and (*c*) coagulation of plasma.

Organoleptic analysis: For each taste panel evaluation two pieces each of fish transported in experimental and control box were cooked and served to a panel of seven members. The coded fish samples were assessed for odour and flavour and the results were scored on a ten-point scale using a score of 5 as the limit of acceptability[6,7].

Results and Discussion

The survey reported here (Table 1) indicates clearly the insanitary condition prevailing with particular reference to transportation system. Considerable variations in microbial load were observed in case of washing water and ice. Microbial load in such case was found to be dependent on the source of water supply. The possibility of contamination of fish during transportation with pathogenic organisms cannot be ruled out as simple water washing of vehicles and containers does not eliminate the contaminating organisms to any appreciable limit. The bacteriological quality of fish transported in returnable and non-returnable containers is reported in Table 2. The result indicates that bamboo basket can help to maintain fair bacteriological standard of fish. Any way, the bacteriological quality of fish transported in experimental container (fibre board) is undoubtedly excellent as compared to fish transported in traditional way. The fishing trade should take more care in handling, transport and distribution of fish. Disinfectant should be used for cleaning the vehicles used for transportation.

TABLE 1. BACTERIOLOGICAL CONDITIONS OF FISH TRANSPORT ENVIRONMENT

Source	No. of tests	Total count	Faecal *Streptococci*	Coliforms	*E. coli*	Coagulase positive *Staphylococci*
		No. of organisms/cc				
1. Washing water	10	$0\text{-}8\times10^4$	$0\text{-}2\times10^4$	$0\text{-}4\times10^3$	$0\text{-}1\times10^3$	—
2. Ice	15	$0\text{-}5\times10^4$	$0\text{-}2\times10^5$	$0\text{-}1\times10^3$	$0\text{-}2\times10^3$	—
		Av. No. of organisms/sq. cm. after usual washing				
3. Vehicles						
Non insulated fish vans	20	9.5×10^8	2.5×10^5	2.5×10^5	2.5×10^3	4.8×10^4
Refrigerated fish vans	20	3.5×10^7	1.2×10^3	2.8×10^3	6.5×10^2	8.5×10^2
Trucks	15	1.9×10^9	3.0×10^5	6.5×10^5	8.2×10^3	5.4×10^4
Carts	10	2.0×10^7	5.5×10^3	8.6×10^2	7.5×10^2	1.5×10^3
4. Containers						
Wooden box	30	1.2×10^8	1.9×10^4	2.5×10^3	120	500
Plywood nox	30	6.2×10^7	8.8×10^3	600	50	300
Plywood box with 1 cm expanded polystyrene insulation	20	1.6×10^4	nil	nil	nil	nil
G. I. Box	25	1.8×10^5	20	"	"	"

TABLE 2. BACTERIOLOGICAL QUALITY OF FISH TRANSPORTED IN DIFFERENT CONTAINERS FROM MADRAS TO CALCUTTA

Type of container	Dimensions (cm)	Ratio of ice to fish	Variety of fish	No. of samples examined	Av. no. of organisms/g of tissue					Sensory rating
					Total count	Faecal *Streptococci*	Coliforms	*E. coli*	Coagulase +ve *Staphylococci*	
Returnable										
Plywood box with 1 cm thick expanded polystyrne in polyethylene bag lining	40×40×35	1:1	Dhoma	20	3.4×10^5	nil	10	nil	nil	7
			Catfish	15	1.1×10^5	6	nil	nil	nil	7
			Mackerel	10	2.0×10^5	nil	nil	nil	nil	7
		Frozen	Dhoma	10	5.0×10^3	,,	nil	nil	nil	7
			Catfish	8	2.3×10^3	,,	nil	nil	nil	7
Plywood box (control)	40×40×35	1:1	Mackerel	12	1.1×10^4	nil	nil	nil	nil	7
			Dhoma	10	5.5×10^6	50	346	12	5	4
			Catfish	10	6.1×10^5	125	210	2	36	4
			Mackerel	10	2.4×10^5	210	853	10	22	5
Non returnable										
Fibreboard box (experimental)	40×40×53	1:1	Dhoma	10	4.5×10^5	nil	nil	nil	nil	5
			Catfish	14	2.5×10^5	nil	12	nil	nil	5
			Mackerel	10	7.0×10^5	nil	nil	nil	nil	5
		Frozen	Dhoma	10	2.2×10^5	nil	nil	nil	nil	7
			Catfish	8	8.2×10^3	nil	nil	nil	nil	7
			Mackerel	12	3.6×10^4	nil	nil	nil	nil	7
Bamboo basket (control)	Irregular	1:1	Dhoma	12	6.7×10^6	10	62	nil	nil	4
			Catfish	15	2.8×10^6	85	125	3	15	4
			Mackerel	10	8.5×10^6	nil	20	nil	nil	4

Acknowledgement

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Studies on Transportation of Wet Fish. III. Biochemical Changes in Relation to Quality of Fish Transported in Fibre Board Container

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Both marine and fresh water fish were transported in fibre board container in iced (1:1 by weight) and frozen state. The fish quality was evaluated after transportation and compared with those transported in conventional bamboo basket (control). Fish can be transported in good condition for 60 hr covering a distance of 1700 km in fibre board (three ply, moisture proof) container and the wood wool used in between two walls of container was found to provide effective insulation.

It is necessary to evaluate the quality of fish by objective and sensory tests. Problems in determining freshness of fish has been described by Stansby[1]. Although several methods are now used to measure spoilage in fish flesh[2-4], until recently none were available for the estimation of shelf life or keeping quality[5]. Many attempts have been made to estimate the storage life of unfrozen fish[6-8]. Shewan and Liston[9] used the ability of bacteria to reduce tetrazolium salts to measure the freshness of iced fish. Spinelli *et al.*[10], Kemp *et al.*[11], Jones *et al.*[12,13], Dugal[14] and Beuchat[15] measured freshness by estimating the extent of nucleotide breakdown in fish muscle cells. It was shown by Lerke *et al*[16]. that the appearance of hypoxanthine in iced fish follows a course similar to that of the direct bacterial count and the latter may be used as freshness test.

Of the instruments the Intelectron Fish Tester Mark V[17] and the Torry Fish Freshness Meter[18] are gaining popularity.

Chemical tests like estimation of hypoxanthine and inosine monophosphate concentrations in addition to organoleptic tests were selected for detailed investigation.

Materials and Methods

Total bacterial count: The procedure is discussed in Part II of this series of papers.

Hypoxanthine in fish muscle: Hypoxanthine concentration in fish muscle was determined by precipitation of the silver salt as described by Jones *et al.*[13] About 20 g of the fish muscle from the dorsal portion was homogenised with 0.6N perchloric acid at 0°C and vacuum filtered. Two millilitre of the filtrate was diluted with 7 ml of distilled water. Silver nitrate solution (0.2 ml of 1M) was added to the diluted filtrate with shaking and precipitate that formed in 15 min at 0°C was separated in a refrigerated centrifuge (Heinzz Janetzki KG, GDR, Model K-24). The supernatant liquid was discarded. The precipitate was washed with 0.1N perchloric acid containing 5 ml of 0.02M silver nitrate and recentrifuged and the washing was discarded. N-hydrochloric acid (15 ml) was stirred vigorously with the precipitate. Silver chloride was removed by centrifugation. Hypoxanthine in solution was estimated spectro photometrically (Zeiss) at 248 nm against a standard hypoxanthine solution (Fluka AG, Switzerland).

Inosine monophosphate (IMP): Preparation of tissue extract-muscle (10 g) from the anterio dorsal portion of the fish was extracted with chilled 3 per cent perchloric acid[19]. The homogenate was filtered and 20 ml were immediately neutralised with 10 per cent potassium hydroxide to pH 6.5. The neutralised extract was stored for approximately 30 min at 0°C to permit crystallization of potassium perchlorate.

Purification of nucleotides: The procedure used by Spinelli and Kemp[19] was followed. Dowex 1-X4(Cl) was washed with 10 per cent ammonium hydroxide and then with 4N hydrochloric acid. Neutralized extract (20 ml) was passed over a 1×2 cm resin bed to separate the nucleotides from nucleosides, purines, pyrimidines and free sugars. Distilled water (35 ml) was passed over the column until the effluent was free from ultraviolet absorbing material. The column was then eluted with 25 ml of 1N sulphuric acid, followed by 5 ml of 6N sulphuric acid. This removed 98 to 100 per cent of the ultraviolet absorbing materials. This was done at 0°-2°C.

Measurement of apparent IMP: An approximate dilution was made of the second column eluate (1:10) with distilled water, so that concentration of the final

TABLE 1. QUALITY OF ICED AND FROZEN FISH TRANSPORTED IN FIBRE BOARD AND BAMBOO BASKET (CONTROL) CONTAINERS

Type of fish	Ratio of ice to fish	Total bacterial count/g $\times 10^5$	IMP μmole /g.	Hypoxanthine μ mole/g.	Sensory rating
After 34 hr of transport					
Dhoma	1:1	4.81	0.41	1.03	6
Control	1:1	62.2	0.28	2.12	6
Mackerel	1:1	6.81	0.76	1.14	6
Control	1:1	11.1	0.69	1.82	5
Bombay duck	1:1	4.20	0.29	0.72	6
Control	1:1	22.8	0.18	1.69	5
Pomfret	1:1	0.432	0.58	1.18	7
Control	1:1	4.25	0.32	2.16	5
Catfish	1:1	0.201	0.62	1.35	7
Control	1:1	0.603	0.43	1.72	6
Dhoma	1:1	1.32	0.38	1.22	6
Control	1:1	22.2	0.32	1.80	5
After 62 hr of transport					
Dhoma	1:1	4.5	0.34	1.52	5
„	Frozen	0.22	0.60	1.23	6
Control	1:1	67	0.22	2.29	4
Catfish	1:1	2.5	0.41	1.80	5
„	Frozen	0.08	0.51	1.48	6
Control	1:1	28	0.18	2.12	5
Mackerel	1:1	7.0	0.72	1.20	6
„	Frozen	0.36	0.81	1.16	6
Control	1:1	85	0.55	2.00	4
Rohu	1:1	5.8	1.11	0.47	6
Control	1:1	11	0.89	0.62	5
Mrigal	1:1	8.2	0.96	0.52	5
Control	1:1	32	0.58	0.85	4
Catla	1:1	6.5	1.07	0.46	6
Control	1:1	22	0.81	0.79	5

solution would be equivalent to 0.1N sulphuric acid. The absorbance of the solution was taken at 250 nm, since at this wave length and acid concentration (0.1N), the adenosine monophosphate (AMP) and IMP have approximately the same molecular extinction coefficient. Apparent IMP was then calculated from a standardised curve prepared by dissolving IMP (Sigma Chemicals, USA) in 0.1N sulphuric acid. All absorbance measurements were made with a Zeiss Spectrophotometer.

Measurement of AMP and actual IMP: The amount of adenosine nucleotides in the column eluate was accurately determined by the method given below:

Sulphuric acid (0.2 ml of 18N) and potassium bromide was (0.1 ml of 0.2M) added to column eluate (2 ml) in a test tube. The solution was shaken for a minute and then 0.3 ml of IN potassium permanganate was added. After 5 min, 6 per cent hydrogen peroxide was added slowly to decolourize excess permanganate. The volume was adjusted to 3.0 ml and after 15 min the colour was read at 330 nm against a reagent blank. A standard was prepared by dissolving adenine (Sigma Chemicals, USA) in 1.8N sulphuric acid containing 0.05M sodium chloride. The amount of actual IMP was calculated by substracting the number of moles of adenine found from the moles of apparent IMP.

Organoleptic analysis: The procedure is as discussed in part II of this series of papers.

Results and Discussion

Quality of fish received in fibre board container and control bamboo basket are given in Table 1. It was observed that wood wool insulation in the fibre board container can keep fish in properly chilled condition.

With the deterioration in quality of fish, the hypoxanthine concentration was found to increase, whereas the inosine monophosphate decreased both in marine and fresh water fish. The result agreed well with the observation made by Kemp and Spinelli[11] during their study with frozen and thawed fish. The transported frozen fish was thawed in running water before experiment.

It can be concluded that both fibre board (nonreturnable) and expanded polystyrene lined used tea chest (returnable) can be used for transportation of fish in iced and frozen condition. It is necessary to transport fish in frozen condition if the distance is more than 1000 km. The capacity of a container should be 50 to 70 kg. The vehicle, reusable container and any other material used in fish transport should be cleaned carefully with disinfectant before each operation.

Acknowledgement

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Microbiological Quality of Frozen Foods Sold in A Nigerian City

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A total of 50 samples of six different types of frozen foods as sold in the stores of a Nigerian city were examined for their microbiological quality. The foods examined were frozen fish, frozen meat, frozen polythene packed meat, processed cheese, frozen chicken and ice cream. Organisms isolated were tested and characterised by standard methods. The following genera were identified: *Staphylococcus*, *Streptococcus*, *Escherichia*, *Achromobacter*, *Flavobacterium*, *Pseudomonas*, and *Alcaligenes*. Mesophilic plate counts showed a range from 4.4×10^3 to 7.0×10^7 org./g whereas in the psychrophilic plate counts the range was from 3.5×10^2 to 9.2×10^5 org./g. *Staphylococci* were present in all the samples, the range being from 1.2×10^2 in ice cream to 4.1×10^4 org./g in frozen fish.

The manufacture and distribution of frozen food was started only about a decade ago in Nigeria. Now a number of companies have facilities for freezing fish, meat, poultry and ice cream and have distribution outlets throughout the federation. Processed cheese slices are, however, imported and sold in a frozen condition. Frozen foods have now become so popular in Nigeria that they are sold even in the local markets. No systematic study has, however, been carried out to assess the microbiological quality of these foods as they are sold to the consumer. At present, the Nigerian Standards Organization is in the process of laying down standards for various foods including frozen foods. This work was therefore, undertaken to evaluate how far the frozen foods being sold in the local markets and supermarkets in Nsukka are microbiologically safe. These data would be of help in setting standards which could reasonably be met. The primary concern is to ensure that the frozen food is safe for human consumption.

Materials and Methods

A total of 50 samples of frozen foodstuffs as sold in the supermarkets of Nsukka were collected as follows: frozen chicken, frozen meat, frozen polythene packed meat, frozen fish, processed cheese all eight samples each and ice cream 10 samples. Immediately on collection, the samples were placed in the freezer in the laboratory. Analyses of the samples were carried out on the day the samples were bought.

Total plate count: Ten grams of each sample was removed when the food was still in a frozen condition using a sterile cork borer and extracted with 0.1 per cent peptone solution for all samples except cheese and ice cream. Cheese samples were emulsified in 1 per cent sodium citrate solution contained in a water bath at 45°C and serial dilutions were made by adding 0.85 per cent NaCl solution.[1] Ice cream samples were serially diluted with Ringer's solution. Plate count agar (Difco) was used for determining plate count of all samples except ice cream where tomato juice agar was used. Plates were incubated at 10° and 37°C for obtaining psychrophilic and mesophilic counts.

Staphylococci: The staphylococci were enumerated by plating the initial homogenates on Baird Parker's

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medium.[2] Plates were incubated at 37°C for 48 hr and typical black, shining, convex colonies 1 to 3 mm in diameter were counted. Typical colonies were confirmed as staphylococci by microscopic examination, gram staining and differentiated from the micrococci on the basis of anaerobic fermentation of glucose.

Haemolytic activity of typical isolates were studied on plates containing nutrient agar overlaid with blood agar, The plasma coagulase test was carried out as described by Evans and Niven[3].

A portion of the initial homogenate was inoculated into selenite F broth[4] and incubated at 37°C for 24 hr. The selenite broth was then inoculated into salmonella and shigella agar to give counts of salmonella.

Presence of coliforms was detected by the MPN method using brilliant green lactose bile broth. Tubes which were positive to both gas and acid were then plated on Eosin—Methylene Blue (EMB) agar. *Escherichia* sp. was detected by the presence of colonies showing dark centres with a metallic sheen.

Other tests such as motility test, catalase test, oxidase test, hydrolysis, agglutination tests, indole test, gelatin liquefaction and fermentation of various sugars were carried out as outlined in Cowan and Steel[5] and Collins and Lyne[6]. The definitions given by Bergey's Manual[7] was also used for identification and characterisation.

Results and Disscussion

The mesophilic and psychrophilic total count in all samples are given in Table 1. The results of mesophilic counts range from 4.4×10^3 org./g for polythene packed meat to 7.0×10^7 org./g obtained for frozen meat. The psychrophilic counts were found to be always lower than the mesophilic counts. The highest psychrophilic count was recorded in frozen meat (9.2×10^5 org./g) while the lowest was recorded in ice cream (3.5×10^2). Staphylococci were found to be present in all the samples analysed, the lowest being in ice cream (1.2×10^2 org./g) whereas the highest was in frozen fish with a value of 4.1×10^4.

TABLE 1. RANGE OF MESOPHILIC, PSYCHROPHILIC AND STAPHYLOCOCCAL COUNT OF SAMPLES OF FROZEN FOODS

	Mesophilic count	Psychrophilic count	Staphylococcal count
	Range (org./g)$\times 10^3$	Range (org./g)$\times 10^3$	Range (org./g)$\times 10^3$
Frozen fish	4.4–129	1–16	2.1 –41
Frozen polythene packed meat	4.4–7300	3.20–560	1.8 –5.6
Frozen meat	104–8420	49–920	1.2 –8.8
Processed cheese	4.98–880	2.00–6.5	0.36–16
Frozen chicken	436–70000	46–690	0.76–23
Ice cream	3.6 –60	0.35–6.5	0.12–6.8

According to Thatcher[8], frozen foods can be considered safe for human consumption if the plate count is not more than 10^5 org./g. Frazier[9] has collected data from several sources to show that a plate count of 2×10^6 to 5×10^6 could be permitted in raw meats. He, however, recommends a limit of the plate count at 10^5 for fish and poultry. Frazier[9] has stated that the U.S. Health Ordinance sets the limits at 5×10^4 for frozen desserts including ice cream. On the basis of the observations cited above, the frozen fish counts of mesophilic bacteria are within the limits. Frozen meat without packaging had in 5 out of the 8 counts more than 5×10^6 org./g which is above the limit recommended by Frazier[9] while packaged frozen meat had only one sample exceeding the limit. In frozen chicken where the recommended limit is 10^5, 3 samples had counts ranging from 10^6 to 10^7. In ice cream, except for one sample which showed a count of 6.0×10^4 all the other samples were below the limit of 5×10^4 set by the U.S. Health ordinance. This was the case with cheese except for one sample. The advantages of packaging for any frozen product is evident from the findings here.

Psychrophilic bacterial counts were on the whole less than the mesophilic counts in all samples. The range observed was from 3.5×10^2 in ice cream to 9.2×10^5 observed in frozen meat. Bacteria belonging to the genera *Pseudomonas*, *Achromobacter*, *Alcaligenes* and *Flavobacterium* were isolated and identified among the psychrophiles. This is in agreement with the observation of Frazier[9] who has reported that in all refrigerated and frozen foods the bacteria usually found are the psychrophiles found here. Slime formation which is characteristic of these bacteria was not observed in any of the samples possibly because the number of organisms were not high enough.

Staphylococci were observed in all the samples. The range of staphylococci was from 1.2×10^2 in ice cream to 4.1×10^4 in frozen fish. All the staphylococci isolated were coagulase positive and some of the isolates haemolysed human red blood cells. All the staphylococci isolated showed positive mannitol fermentation. This is in agreement with the observations made by Joshi and Dale[10]. The presence of staphylcoccci always presents a potential danger of enterotoxin formation.

Table 2 gives the frequency of isolation of staphylococci, coliforms, streptococci and salmonellae. Staphylococci was the one most frequently found. This is in agreement with the findings of Georgala and Hurst[11], who postulated that staphylococci being gram positive organisms are more resistant to freezing temperatures than the gram negative ones. *Escherichia* sp. was absent

TABLE 2. ISOLATION OF VARIOUS MICROORGANISMS FROM DIFFERENT FROZEN FOOD SAMPLES

	No. of samples in which specific genera were found					
	Frozen fish	Frozen meat	Polythene packed frozen meat	Cheese	Frozen chicken	Ice Cream
Escerichia sp.	2	2	—	—	1	—
Staphylococcus sp.	8	8	8	8	8	10
Streptococcus sp.	3	4	2	3	3	2
Salmonella sp.	3	3	1	—	2	—

Total no. of samples examined is 8

in polythene wrapped meat, cheese and ice cream and the frequency of occurrence in other products was also low. Salmonella was absent in cheese and ice cream. The frequency of occurrence of salmonella was highest in frozen fish and frozen meat and in only one sample of wrapped frozen meat was salmonella found. The presence of coliforms, streptococci and salmonellae and staphylococcci indicates poor handling. It would be necessary to find out at what stage these products get contaminated and devise procedures to prevent the entry of these microorganisms.

Conclusions: As most of the samples examined did not conform to specifications for total count and other counts such as stapylococci, more analyses would have to be done both at the factory level during various stages of processing and at retail outlets to find out where the contamination sets in. This would help in (*a*) discovering the sources of contamination and taking steps to prevent it. (*b*) Knowing the limits set for microbiological standards for the various frozen foods marketed in Nigeria.

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Growth and Toxicogenesis of *Clostridium botulinum* Type E on Marine Molluscs at Low Temperatures

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The spores of Minneapolis and 1304 of *Clostridium botulinum* type E were inoculated (approximately 1×10^4 spores) into fresh Pacific oysters and clams, which were then incubated anaerobically at 13°, 10°, 7°, 4° and 2°C. During incubation the salt content was reduced, the pH values dropped and the acidity increased. After 90 days of incubation, toxin production was tested by injecting intraperitoneally into white Swiss Webster mice. Both strains of *Clostridium botulinum* type E produced toxin at 13°, 10°, 7° and 4°C but not at 2°C. Similarly, in heat sterilized oysters and clams, toxin was found after 90 days at all incubation temperatures except 2°C. The strain Minneapolis was found to be more active in growth as well as in toxin production than strain 1304.

Oysters and clams are of extreme economic importance in the Pacific coast area. They are not as adaptable to freezing as other shellfish, so the major part of these products are marketed fresh or chilled. Since oysters and clams, when packaged for marketing, contain considerable numbers of bacteria, including public health hazard organisms, the maintenance of conditions which will prevent bacterial increases during transit, distribution and on the retailer's shelf is a problem of major importance.

Schmidt *et al*[1]. reported that some strains of *Clostridium botulinum* type E can form toxin slowly at 3.3°C. The 1963 outbreaks of botulinum food poisoning resulting from the ingestion of type E botulinal toxin in smoked whitefish and smoked whitefish chubs from the Great Lakes[2,3,4], as well as earlier outbreaks aroused interest in the incidence of *Clostridium botulinum* type E in other marine species such as salmon, tuna, shrimp, crab, clams and oysters. Bott *et al*[5]. reported that *Clostridium botulinum* type E is part of the natural flora of the fish of the Great Lakes. Other workers have also reported a widespread distribution of *Clostridium botulinum* type E in both fresh water and marine fish[6,7].

The new methods of packing foods, such as the plastic wrapping materials for food, which are impermeable to oxygen and vapour, may be conducive to the growth and toxin production of *Clostridium botulinum* type E. Usually, the oysters and clams are marketed in tightly sealed containers and during long storage the entrapped oxygen may be absorbed and utilized by the tissues of oysters and clams. Thus, eventually this provides a favourable condition for the growth of anaerobic bacteria like *Clostridium botulinum* type E. Moreover, a few cases of aerobic, or apparently aerobic production of toxin by these organisms have been reported[8].

Information is not available as to whether the *Clostridium botulinum* type E spores can grow and produce toxin in contaminated oysters and clams in competition with other bacteria present as natural flora, especially at low temperatures. An attempt is made in the present study to determine the growth and toxicogenesis of two strains of *Clostridium botulinum* type E in raw as well as sterile oysters and clams at low temperatures.

Materials and Methods

Substrate: The chilled fresh Pacific oysters (*Crassostrea gigas*) and clams obtained from Meredith Fish Company, Sacramento, were used as substrate. Also Pacific oysters and clams sterilized at 121°C for 15 min were used as substrate.

Test organisms: The organisms used in this experiment were the strains Minneapolis and 1304 of *Clostridium botulinum* type E.

Preparation of spore suspensions: The test organisms were subcultured by inoculating the stock culture into 10 ml sterilized liver infusion broth tubes (beef liver infusion, 50 per cent; proteose peptone, 1 per cent, and NaCl, 0.5 per cent) with meat particles; and incubating them at 30°C for 48 hr. The tubes of trypticase (5.0 per cent), proteose peptone (0.5 per cent), glucose (0.4 per cent), yeast extract (1.0 per cent), $(NH_4)_2SO_4$ (1.0 per cent) and sodium thioglycollate (0.1 per cent), pH 7.2 (TPGY) broth were inoculated from these subcultures and incubated at 30°C for 24 hr. Using these broth cultures, the spore suspensions were prepared according to the biphasic culture technique procedure[9].

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The development of the spores was followed using a Petroff-Hauser counting chamber with phase microscopy. The peak refractile spore count occurred after 24-40 hr of incubation. Spore sediments were obtained by centrifugation at 1,000 $\times$g for 60 min at 4°C, washed twice with sterile physiological saline and once with 65 per cent ethanol.

Dilution and inoculation: With total count of spore suspension used as a guide, viable count was made using serial dilution method with liver infusion agar plus 0.14 per cent $NaHCO_3$ and 0.1 per cent sodium thioglycollate which were immediately incubated under anaerobic conditions at 30°C for 48 hr. Proper dilution of the spore suspension was made to give 1.0×10^4 spores/ml using the viable count as base. Approximately 70-80 g of oysters and clams were put into screw capped bottles. Each bottle was inoculated with about 1.0×10^4 spores and was covered with sterile mineral oil.

Incubation and measurements: 24 bottles of raw oysters and raw clams, and 4 bottles of sterile oysters and sterile clams were incubated at each temperature of 13°, 10°, 7°, 4° and 2°C for 90 days. At the interval of every 15 days, measurements of pH, acidity, salt content and anaerobic counts were made in duplicate samples.

Measurement of pH: The pH was measured with the glass electrode (Beckman, Expandomatic SS-2 model).

Measurement of per cent acidity: The per cent acidity expressed as lactic acid was obtained by titrating 5 ml fluid of oysters and clams against 0.1N sodium hydroxide solution.

Measurement of per cent salt content: The per cent salt content was obtained by titrating 1 ml fluid of oysters and clams against 0.171 N silver nitrate solution using dichlorofluorescein as an indicator up to the end point, when permanent salmon pink coloured precipitates were formed.

Toxin assay: The fluid of oysters and clams was used for toxin assay. Trypsin digestion was carried out by adding sterile crude commercial trypsin to a final concentration of 1 per cent into sample fluid, and incubating it at 37°C for 1 hr. After this, 0.5 ml portions of fluid were injected intraperitoneally into White Swiss Webster mice, weighing 20-23 g. Also 0.5 ml of boiled sample was injected into white mice to serve as a heat-labile toxin control. 0.5 ml of sample fluid, mixed with 0.1 ml type E antitoxin and held at room temperature for 30 min, was injected into white mice to demonstrate the presence of specific type E toxin.

Growth curve: Liver infusion broth (Difco) plus 0.1 per cent sodium thioglycollate was used. The optical density was measured with B and L Spectronic-20 spectrocolorimeter at 520 nm.

Results and Discussion

One of the physiological features of *Clostridium botulinum* type E, is the ability to grow at low temperatures. Schmidt *et al*[1]. reported that mildly heat-shocked spores of four strains of *Clostridium botulinum* type E germinated and produced toxin in heat-sterilized beef stew substrate within 30-45 days of the incubation at 3.3°C but not at 1° and 2°C. Fig 1 shows the growth curves of two strains of *Clostridium botulinum* type E under anaerobic conditions (nitrogen gas) at various temperatures. At 13° and 10°C, strain Minneapolis showed maximum growth a little earlier than strain 1304, while at 7°C maximum growth of strain Minneapolis occurred several days earlier than that of strain 1304. At 4°C both strains generally showed the same pattern of growth, but the maximum growth was markedly delayed. No growth was observed at 2°C even after 60 days of incubation.

Table 1 shows the data on the quality of the fresh Pacific oysters and clams. There were differences in salt content of both species. The microbiological analysis showed that they carry both psychrophilic as well as anaerobic bacteria among their natural micobial flora. The clams seemed quite fresh, but the oysters might have been a few days old as the acidity found was slightly higher than normal.

Changes in pH (Table 2) and acidity (Table 3) were observed during incubation. The pH values decreased in Pacific oysters and clams at all low temperatures, even under anaerobic conditions[10,11]. As the incubation temperature decreased from 13° to 2°C, the rate of pH drop also decreased. At 13° and 10°C, on an average, the same rate of pH drop was observed, but it was quite rapid as compared to the other low incubation temperatures. At 2°C pH drop was definitely slow. At 13° and 10°C the lowest pH values occurred

TABLE 1. QUALITY OF FRESH PACIFIC OYSTERS AND CLAMS

Analysis	Material	
	Pacific oysters	Clams
Total count per ml on G.T.Y. at 30°C for 48 hr.	4.0×10^4	1.4×10^4
Psychrophilic count per ml on G.T.Y. at 2°C for 15 days	8.0×10^3	8.5×10^3
Anaerobic count per ml on liver infusion+0.1% sodium thioglycollate at 30°C for 48 hr.	7.0×10^4	8.2×10^4
pH	5.75	6.30
% acidity (expressed as lactic acid)	0.72	0.34
% salt content	1.0	2.4

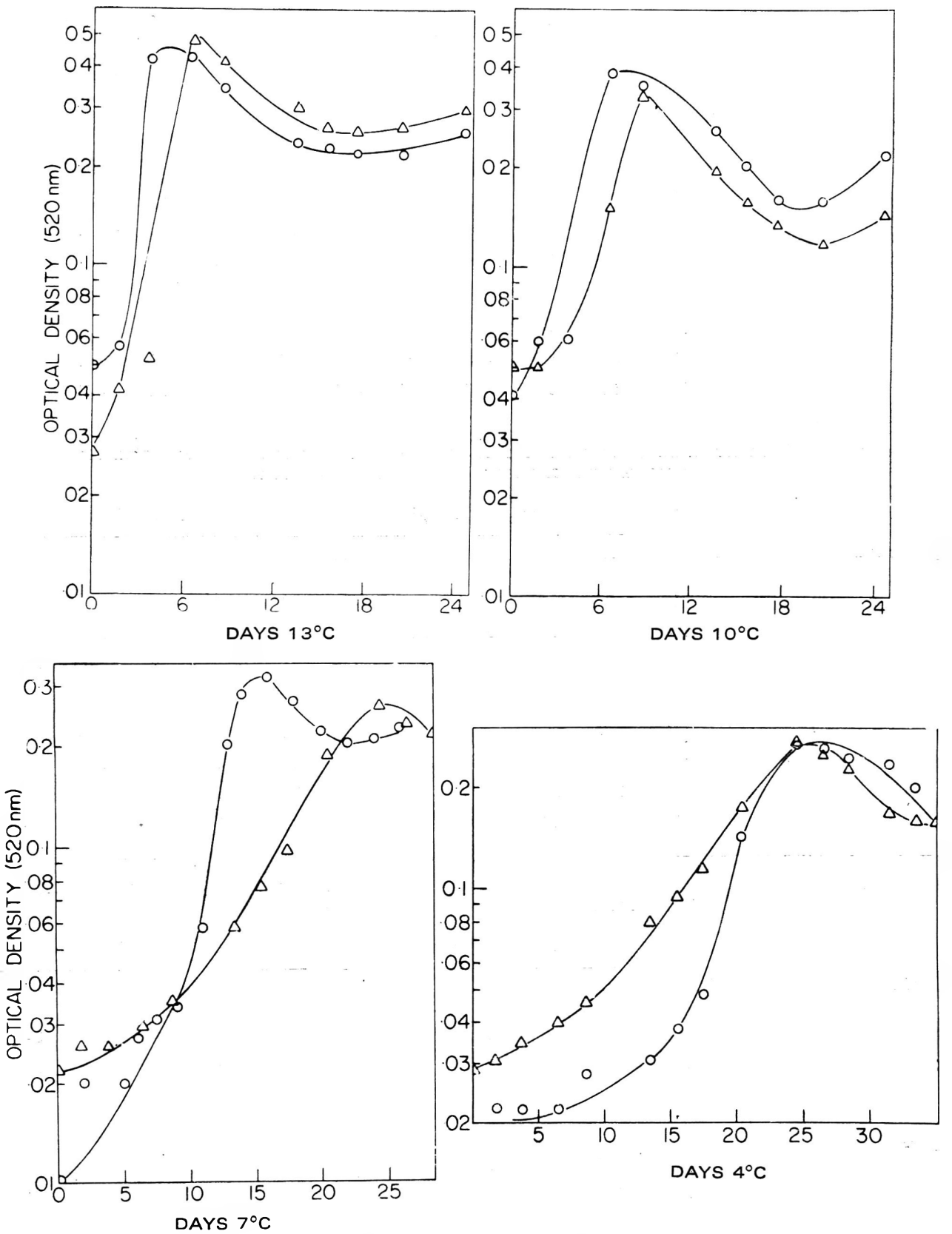

Fig. 1. Growth curves of *Clostridium botulinum* type E at various low temperatures

TABLE 2. CHANGES IN pH IN FRESH PACIFIC OYSTERS AND CLAMS INOCULATED WITH SPORE SUSPENSION OF *CLOSTRIDIUM BOTULINUM* TYPE E, AT LOW TEMPERATURES

		Pacific Oysters at °C					Clams at °C				
Type of strain	No. of days	13	10	7	4	2	13	10	7	4	2
Minneapolis	0	5.75	5.75	5.75	5.75	5.75	6.30	6.30	6.30	6.30	6.30
	30	4.25	4.33	4.75	4.80	5.70	.490	5.35	5.32	5.50	5.75
	60	4.50	5.00	4.45	4.61	4.60	5.20	4.80	4.90	4.98	5.40
	90	5.00	5.20	4.90	4.20	4.12	5.40	5.31	5.61	4.75	4.80
1304	0	5.75	5.75	5.75	5.75	5.75	6.30	6.30	6.30	6.30	6.30
	30	4.40	4.50	4.60	5.10	5.30	5.00	5.00	5.20	5.42	6.00
	60	4.45	5.15	4.45	4.60	4.85	4.80	4.80	4.90	5.15	5.20
	90	5.25	5.25	4.90	4.10	4.10	5.20	5.35	5.29	4.80	4.91

TABLE 3. CHANGES IN % ACIDITY (EXPRESSED AS LACTIC ACID) IN FRESH PACIFIC OYSTERS AND CLAMS INOCULATED WITH SPORE SUSPENSION OF *CLOSTRIDIUM BOTULINUM* TYPE E, AT LOW TEMPERATURES

		Pacific Oysters at °C					Clams at °C				
Type of strain	No. of days	13	10	7	4	2	13	10	7	4	2
Minneapolis	0	0.72	0.72	0.72	0.72	0.72	0.34	0.34	0.34	0.34	0.34
	30	3.74	3.44	2.41	2.09	1.53	3.24	2.34	2.40	2.11	1.89
	60	3.42	2.95	2.79	2.52	2.57	2.61	3.06	2.92	2.59	2.39
	90	2.39	2.66	2.18	3.17	3.28	2.25	2.38	1.98	2.75	2.66
1304	0	0.72	0.72	0.72	0.72	0.72	0.34	0.34	0.34	0.34	0.34
	30	3.83	3.73	2.92	2.39	1.04	2.86	2.86	2.48	1.96	1.35
	60	3.80	2.41	3.67	2.92	2.81	3.15	2.83	2.99	2.32	2.45
	90	2.41	2.32	3.10	3.17	3.28	2.57	2.38	2.34	2.97	2.92

TABLE 4. CHANGES IN % SALT CONTENT IN FRESH PACIFIC, OYSTERS AND CLAMS INOCULATED WITH SPORE SUSPENSION OF *CLOSTRIDIUM BOTULINUM* TYPE E, AT LOW TEMPERATURES

		Pacific Oysters at °C					Clams at °C				
Type of strain	No. of days	13	10	7	4	2	13	10	7	4	2
Minneapolis	0	1.00	1.00	1.00	1.00	1.00	2.40	2.40	2.40	2.40	2.40
	30	0.65	0.68	0.65	0.65	0.65	1.55	1.65	1.65	1.75	1.85
	60	0.65	0.65	0.70	0.70	0.65	1.45	1.55	1.60	1.65	1.75
	90	0.60	0.65	0.70	0.75	0.75	1.70	1.70	1.70	1.60	1.55
1304	0	1.00	1.00	1.00	1.00	1.00	2.40	2.40	2.40	2.40	2.40
	30	0.70	0.70	0.65	0.65	0.65	1.65	1.65	1.70	1.70	1.90
	60	0.65	0.65	0.70	0.70	0.65	1.40	1.60	1.60	1.60	1.65
	90	0.70	0.70	0.60	0.60	0.75	1.60	1.70	1.55	1.48	1.60

after 30 days incubation, and at 7°C the lowest pH values occurred after 60 days incubation. But the lowest pH values at 4° and 2°C were during the last stage of incubation.

Thus, lowering of pH occurred more at 2° than at 13°C, which might be due to the presence of natural microbial flora such as *Pseudomonas*, *Micrococcus*, *Proteus* and coliform groups in shellfish. Wilson and McClesky[12] found that coliform organism readily developed when shucked oysters were stored at 3°-4°C. Initially the increase was slow, but enormous numbers were encountered after 4 weeks. It is suggested that the bacterial cause of drop in pH values at low temperatures as well as under anaerobic conditions might be due to the activities of these organisms. Conversely, increases in acidity occurred along with decreases in pH values. The acids are formed from the breakdown of glycogen known to be distributed throughout the oysters. This breakdown is presumed to be due to bacterial action and/or glycolytic enzymes within the oyster tissue[13].

The salt content was also reduced during incubation (Table 4). At all temperatures sharp reduction in salt content was observed in the first fifteen days' incubation period, but from then on it reduced slowly. The reduction in salt content presumbly may be due to binding of salt within the muscles of oysters and clams, thus leaving less salt content in fluid which was usually tested after stirring. It may also be due to a slight dilution effect by the inoculum. These results are similar to those which are usually observed in sauerkraut fermentation, where salt is important for firmness of the product.

The anaerobic count increased from 10^4 to 10^8/ml within 15 days of incubation at 13° and 10°C, but from then on it progressively decreased. At 7°C the highest anaerobic count was observed during 30-45 days of incubation (10^8/ml), but at 4° and 2°C the highest anaerobic count was found after 45 days. During the last stage of incubation, the anaerobic count was low at 13°, 10° and 7°C (10^5-10^6/ml) as compared to 4° and 2°C (10^7-10^8/ml in oysters and 10^6-10^7 in clams) but a definite drop was observed at all temperatures. Similar results for plate counts and coliform MPN were noted by Hoff *et al*[10]. in Pacific and Olympia shucked oysters stored in ice and at 3° and 10°C.

Schmidt[1] and other workers[3,8] have stressed the importance of *Clostridium botulinum* type E as being tolerant to such unfavorable environmental conditions as low temperature, low pH, and high salt or sugar content. In this experiment the initial pH of oysters and clams was favourable for the growth of *Clostridium botulinum* type E strains. Moreover, the salt content of both the species was too low to inhibit or delay the outgrowth and toxin production by inoculated spores of *Clostridium botulinum* type E. The fermentable carbohydrate is present in sufficient amount in oysters and clams, particularly oysters, which contain large amounts of glycogen, which helps in germination, growth and toxin production by type E spores.

To identify the production of type E toxin, mice were injected intra-peritoneally as described earlier, and the findings regarding the production of toxin were observed by means of the death or survival of mice. At all incubation temperatures except at 2°C, spores of *Clostridium botulinum* type E germinated, grew and produced toxin in competition with the naturally occurring microbial population, as revealed by the death of mice.

The death of mice occurred usually within 2-6 hr, indicating heavy production of toxin by *Clostridium botulinum* type E. This suggests that production of toxin might have occurred even prior to completion of the 90 days' incubation period. Similar findings were also observed regarding toxin production by *Clostridium botulinum* type E; in heat-sterilized Pacific oysters and clams, i.e., in a non-competitive environment, at the same incubation temperatures.

This presents the threat of type E botulism from a public health point of view. Many workers have reported the presence of *Clostridium botulinum* type E in shellfish. Also, chances of mishandling of shellfish during transportation as well as by retailers and consumers, are ever present. Thus, shellfish harbouring type E spores might become a cause of type E botulism if kept at temperatures only a little higher than refrigeration for a comparatively long period.

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Microbial Flora of Dehydro-irradiated Shrimps

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Fresh shrimp was dipped in sorbic acid, blanched and dehydrated to 40% moisture, before subjecting to irradiation at 250 Krad. After irradiation the total bacterial count was reduced from 1.1×10^5 to 1.4×10^2 cells/g and the total anaerobic count from 9.5×10^4 to 1.3×10^3 cells/g. In irradiated shrimp *Bacillus* sp predominated followed by *Micrococcus* sp.

Combination treatments of heat and gamma radiation have been reported to stabilize shrimps for ambient temperature storage. The integrated processes involve a dip of peeled and deveined shrimps in 0.5 per cent sorbic acid for 60 min, blanching in 10 per cent NaCl solution at 80°C for 5 min, dehydration to 40 per cent moisture and irradiation at 0.25 Mrad[1]. The treated shrimp containing 25 mg per cent sorbic acid and 3.25 g per cent of NaCl have been found to remain free from mold and bacterial spoilage for 4 months at room temperature (25-28°C). The product possessed better organoleptic properties and higher rehydration capacity in comparison to commercially available dried shrimps. Although detailed studies have been made in our laboratory on the storage stability and packaging aspects[1,2], information is lacking on the microbial flora of the product. The present communication reports on the microbial profiles in shrimp before and after processing.

Fresh shrimp (*Penaeus indicus*) purchased from a local market were brought to the laboratory in ice. The shrimps were washed in running tap water, peeled, deveined and again washed thoroughly in potable water. Shrimps were dipped in sorbic acid solution, blanched and dehydrated to 40 per cent moisture level in a laboratory model air-drier at 55°C. The details of the process have been described earlier[3]. The treated shrimps were packed in polycell bags (325 g low density polyethylene 300 MST cellophane), sealed and irradiated at 250 Krad using a ^{60}Co Package Irradiator (Atomic Energy, of Canada Ltd.) at a dose rate of approximately 200 Krad/hr. For total bacterial counts 10 g of the processed shrimp were blended with 90 ml sterile 0.2 per cent peptone (Difco). Total counts were determined by the pour plate method using nutrient agar (Difco), after incubation at 28°C for 48 hr. For purpose of identification of the bacterial flora, about 50 per cent of the colonies on the plates were selected. The method used for characterisation of the different isolates was based on procedures reported by Shewan *et al*[4]. and Masurovsky *et al*[5]. Standard media[6] were used for assessment of indole production, nitrate reduction, gelatin liquefaction and sugar fermentation. Catalase activity was tested by flooding each colony with a 3 per cent solution of hydrogen peroxide and observing the evolution of oxygen.

Table I presents the microbial spectra of the processed shrimp before and after irradiation. The initial aerobic viable count of the unirradiated sample was 1.1×10^5 cells/g. After exposure to 0.25 Mrad, the total count was reduced by approximately 3 log cycles to give a value of 1.4×10^2 cells/g. The irradiation treatment also reduced the total anaerobic count from 9.5×10^4 to 1.3×10^3 cells/g. The irradiated samples were found to be free from molds, *Staphylococci* and *Enterobacteria*.

Of the organisms isolated from unirradiated processed shrimps, 96 per cent were catalase positive, and 22 per

TABLE 1. EFFECT OF PROCESSING TREATMENTS ON THE COMPOSITION OF THE MICROBIAL FLORA OF DEHYDRO-IRRADIATED SHRIMP

Microbial groups	Processed shrimp before irradiation	Processed shrimp after irradiation
Micrococcus	68	36
Staphylococcus	20	nil
Bacillus	2	42
Aeromonas	6	nil
Coryneforms	2	12
Unidentified	2	10
Total isolates	49	15
Aerobic count/g	1.1×10^5	1.4×10^2
Anaerobic count/g	9.5×10^4	1.3×10^3
Mold count/g	9.0×10^1	nil

The numerical values assigned to microbial groups are expressed on a percentage basis, calculated from the total number of isolates of respective samples.

cent were capable of liquefying gelatin. It was observed that the processing of shrimp prior to irradiation resulted in almost complete elimination of gram-negative bacteria which are known to be active spoilers in fishery products. Of the gram-positive flora, *Micrococcus* was found to be the most predominant group present in processed unirradiated shrimp. Other bacteria included *Bacillus* sp. and *Coryneforms*. Irradiation of the processed shrimp resulted in a shift in the microbial pattern. It was found that in irradiated shrimp, *Bacillus* sp. predominated, followed by *Micrococcus* sp. These results are in agreement with earlier reports[3,9]. Also *Micrococcus* and *Bacillus* have been reported to be the major suvivors in Bombay duck and haddock fillets[3,5]. Since *Micrococcus* sp. are not responsible for putrefaction of sea-foods, the spoilage of the dehydro-irradiated shrimp could occur when spores of the predominant *Bacillus* germinate and outgrow in the product. It was however, observed that *Bacillus* spores isolated from semi-dried shrimps did not germinate in the presence of glucose or l-alanine indicating their cryptodormant character[9]. Therefore, the limiting factor in determining the shelf-life of dehydro-irradiated shrimp could be attributed to chemical changes rather than microbial spoilage of the product.

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Studies on Biochemistry of Higher Fungi. II. Submerged Growth of A Few Mushrooms in Synthetic Media

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Three wild mushroom strains, *T. clypeatus*, *P. papillionaceus* and *G chrysimyces* grow well in defined media containing carbohydrate and simple mineral salts. Their growth is not well supported by glucose but hightly stimulated by glucose polysaccharides. $NH_4H_2PO_4$ and urea as nitrogen sources support growth well in presence of polysaccharide. They have at least two C/N ratios for optimal growth, one being common at 5 and others at 10, 15 and 26 respectively. Trace elements have effect not only on the growths but also on the flavour and pigmentation of the broths. The pH optima are 2.5, 2.5 and 5.0 respectively and showed acid reaction throughout the course of fermentation. Their protein content ranged from 27.23 to 31.76, fat, 1.0 to 5.15: carbohydrate, 34.7 to 52; fibre, 10.5 to 24.25 and ash, 0.5 to 5% (dry weight basis).

Submerged propagation of mushroom mycelium was initially developed for the steady production of edible mushrooms for human consumption. But during the last twenty years, several industrial and medical applications of the mushroom fermentation have been investigated *viz.* production of enzymes,[1,2], carcinostatic polysaccharides,[3] antibiotics[4], nucleotides[5], pharmacologically important compounds[6], etc. Although from time to time it has been reported[7-9] about the submerged cultivation of various mushrooms studies were mainly concentrated on the production of mycelial bodies. Knowledge regarding biochemical studies on their nutritional requirements, growth patterns and biochemical changes taking place during fermentation, of mushrooms particularly in fully defined media is rather meagre.

This paper describes some biochemical studies on the fermentation of a few mushroom strains, collected from Indian soil and grown in completely defined media.

Materials and Methods

Mushrooms: Strains were isolated from the inner part of young fruit bodies of mushrooms collected from

the local fields. The fruit bodies were identified as the strains of *Termitomyces clypeatus* (Heim) *Panafolus papillionaceus* (Bull. ex. Fr.) *Quel* and *Gymnopilus chrysimyces* (Berk).

Chemicals: For synthetic and semisynthetic media, analytical grade chemicals were used. Straw lignin[10] was prepared from washed straw by extraction with 1 per cent NaOH for 2 hr at 95°, followed by precipitation with acid, washing with water and finally boiling with 2 per cent HCl for hydrolysis of contaminated cellulose or hemicellulose. The product is finally washed and dried under vacuum. Malt extract was purchased from Oxoid Laboratory, England, and potato extract was prepared in the laboratory from fresh potato by extracting fresh peeled potato with boiling water for 45 min and filtering the extract. For the studies of micronutrient requirements, special precautions were undertaken to avoid all chances of metal contamination. Highly cleaned corning glass wares and triple glass distilled water were used throughout this investigation.

Initial propagation in complex medium: Fresh fruiting bodies of mushrooms, after collection from fields, were lightly washed with absolute alcohol and small pieces from the inner body were cut aseptically and inoculated on solid agar medium (15 ml/250 ml conical flask). The complex media used contain (w/v per 100 ml); glucose, 5; malt extract, 1; potatoe xtract, 10; $CaCl_2$, $2H_2O$, 0.037; KH_2PO_4, 0.087; $MgSO_4$, $7H_2O$, 0.025; $MnCl_2$, $4H_2O$, 0.0036; $NaMoO_4$, $4H_2O$. 0.0032; $ZnSO_4$, $7H_2O$, 0.03;, pH, 5.0; agar, 2.5. Temperature was always maintained between 28° and 32°C. Mycelial growth was observed after 40-48 hr and rapid filament elongation continued upto 7-8 days. The strains were further purified by subculturing from the tips on the same media containing streptomycin (30 μ g/ml) and mycobacillin (20 μ g/ml), the two broad spectrum antibacterial and antifungal antibiotics. Strains were maintained in the same medium.

For submerged propagation, mycelial growth from each flask was totally transferred to the same liquid medium (100 ml/500 ml flask) and put on a rotary shaker (350 rpm and throw of 3.5 cm) for 15 days. Mycelial balls appeared after 2-3 days of inoculation and fruity flavour, characteristic of mushroom growth appeared after 7 to 8 days. Typical mushroom mycelial morphology was also checked time to time under microscope. In each experiment, growth, always in triplicate, was measured 15 days after inoculation.

Nutrient studies: The mycelial pellets after 15 days of submerged growth were washed free of broth and suspended in 100 ml sterile water in a Waring blender and cut into small pieces. All the operations were done under aseptic condition. This suspension (2 ml) was used as inoculum per 100 ml of liquid media.

For investigations on their nutritional requirements, the basal media were derived from the complex medium used for inital propagation of mushrooms. Carbon source glucose, was serially replaced by dextrin, soluble starch, amylum, lignin[10] and soluble carboxymethyl cellulose. Potato and malt extract, the only ingredients providing various nitrogen sources in the complex medium were replaced by various inorganic nitrogen sources. After selection of proper carbon and nitrogen sources of the media, concentrations of both the sources were varied to obtain C/N ratio for best growth.

In the complex medium, different micronutrients, as reported by various workers[11,12], required for mushroom growth, were included initially and then selections were made by omitting them serially from the media containing selected carbon, nitrogen sources and KH_2PO_4, $MgSO_4$ and the resulting effect of omissions on the growth were studied.

The mycelial balls after 15 days of growth, were filtered, washed, and weighed after dehydration at 70-80°C for 48 hr.

Optimum pH, growth curve and biochemical analysis of mycelial growth: Optimum initial pH for growth and growth curves for the mushrooms were determined by the usual methods. Mycelial bodies at their maximum growths were assayed for protein by Micro-Kjeldahl method and fibre[13], fat[13], ash[13], carbohydrate[14] by standard methods.

Results

Influence of various carbon and nitrogen sources: The original complex medium, containing various complex ingredients and glucose, was initially altered by the replacement of glucose with various polysaccharides. It appears from Table 1, that polysaccharides excellently stimulate their growth compared to glucose. Thus dextrin and soluble starch are excellent substitutes for glucose. In the media, at present the nitrogen nutri-

TABLE 1. EFFECT OF SUBSTITUTION OF GLUCCSE BY VARIOUS, CARBON SOURCES

Carbohydrate (5% W/V)	Av dry wt of mycelium (g/100 ml)		
	T. clypeatus	*P. papillionaceus*	*G. chrysimyces*
Glucose	0.063	0.115	0.100
Dextrin	1.779	0.456	0.880
Soluble starch	1.613	1.167	1.407
Carboxymethyl cellulose	0.095	0.093	0.055
Straw lignin	0.213	0.333	0.200
Amylum	0.505	0.323	0.981

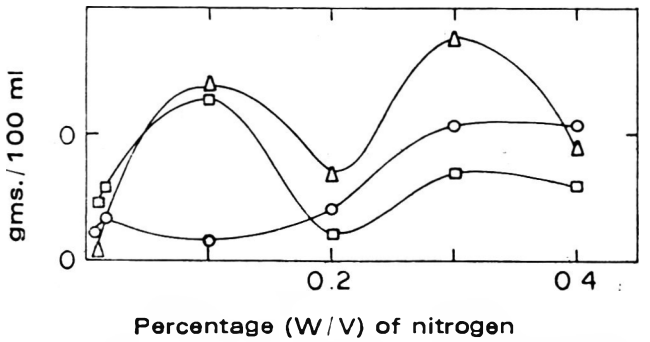
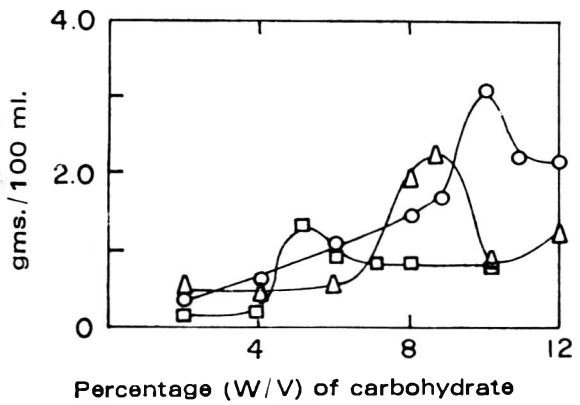


Fig. 1. Mycelial yields under various nitrogen concentrations; O-O-O, *P. papillionaaceus;* △-△-△, *T. clypeatns; and* □-□-□ *G. chrpsimpees.*

ents were provided by malt and potato extract which were subsequently replaced by various simple inorganic nitrogenous compounds including urea. It is seen from Table 2, that among the various nitrogen sources tested (at the same nitrogen content), $NH_4H_2PO_4$ supports growth better for *T. clypeatus* and *P. papillionaceus*, while *G. chrysimyces* utilises urea. With the replacement of malt extract and potato extract by only inorganic nitrogen salts, growth was slightly diminished for two strains but it was increased for *P. papillionaceus.*

Mycelial yields with variation of nitrogen, carbon concentrations and C/N ratios: With the selection of carbon and nitrogen sources (Table 1 and 2), in comparison to glucose and complex nitrogens, their optimum concentrations were determined by growing them at various nitrogen concentrations at a fixed carbon concentration (5 per cent w/v) and then varying the carbon concentrations at the best nitrogen concentrations as observed in the previous experiment. Results are presented in Fig. 1 and 2. It is clear from Fig. 1, that yields of mycelia with the variation in nitrogen concentration ($NH_4H_2PO_4$ for both *T. clypeatus*, *P. papillionaceus* and urea for *G. chrysimyces*), exhibit two peaks which are very similar for *G. chrysimyces* and *T. clypeatus*; but for *P. papillionaceus*, first peak is less marked



F.g. 2. Mycelial yields under various carbohydrate concentrations; O-O-O. *T. clypeatus;* △-△-△, *P. papillionaceus and* □-□-□, *G. chystmyces.*

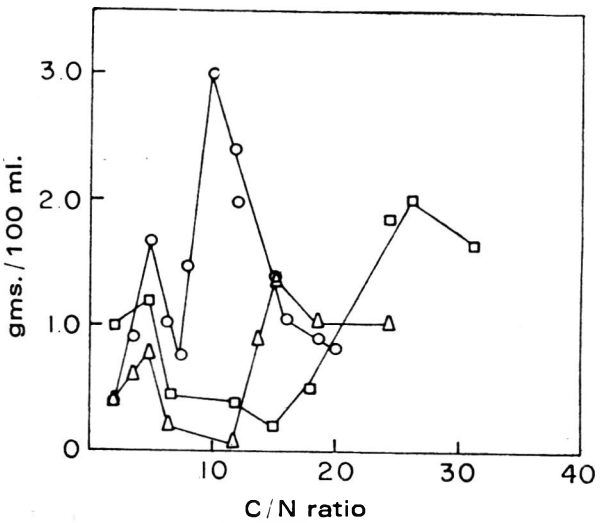


Fig. 3. Mycelilal yields at various C/N ratios. △-△-△, *G. chrysimyces;* O-O-O, *T. clypratus.* □-□-□, *P. papillionaceus.*

and occur at very low nitrogen concentration. On the basis of yields, nitrogen concentration corresponding to second peak was considered to be optimal. The effect of variation of carbon concentration, (soluble starch for *P. papillionaceus*, *G. chrysimyces* and dextrin for *T. clypeatus*) at these optimal nitrogen concentrations, on the growths are presented in Fig. 2. The best carbon concentration either as dextrin or as soluble starch are found to be 5, 9 and 10 per cent (w/v) respectively for *G. chrysimyces*, *P. papillionaceus* and *T. clypeatus.*

These variations of carbon and nitrogen concentrations in the synthetic medium and their resulting effects of growth also gives a series of C/N values and corresponding growths. This is presented in Fig 3.

Micronutrient make up in relation to growth, pigmentation and flavour production: It has been observed that during the last phase of mushroom fermentation in complex medium, there is always production of sweet

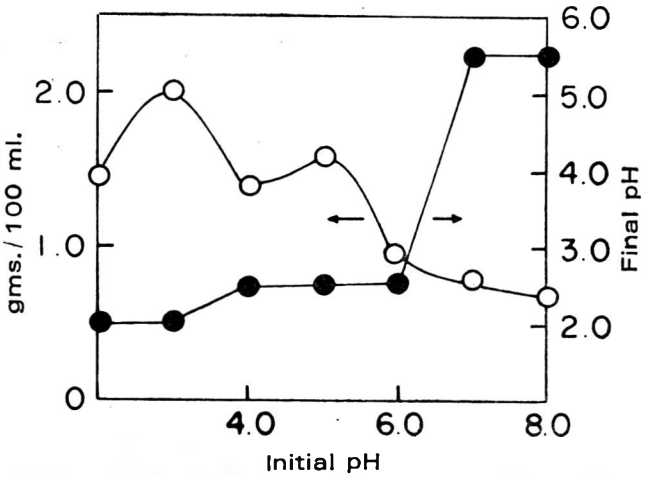


Fig. 4. The influence of initial pH on the mycelial yield of *T. clypeatus*, ●-●-●, final pH; O-O-O, mycelial dry weight.

TABLE 2. EFFECT OF VARIOUS NITROGEN SOURCES IN THE SYNTHETIC MEDIA

Organism	C. sources at 5% (W/V)	Av. dry mycelial wt. (g/100 ml) for the N sources					
		NH_4NO_3 0.3% (W/V)	$(NH_4)_2SO_4$ 0.5% (W/V)	KNO_3 0.73% (W/V)	$NH_4H_2PO_4$ 0.9% (W/V)	Urea 0.22% (W/V)	NH_4Cl 0.4% (W/V)
T. clypeatus	Dextrin	0.917	1.507	0.748	1.687	0.050	0.334
P. papillionaceus	Soluble starch	1.448	0.173	0.109	1.537	0.362	0.101
G. chrysimyces	,,	0.922	0.203	0.185	0.231	0.959	0.308

Glucose, potato and malt extracts are omitted from the culture medium.

fruity smell which is not always obtained in synthetic media. Micronutrient compositions of synthetic media have marked effect on the production of fruity flavour. Thus, omission of Zn^{++} or all micronutrients causes flavour production during the growth of *T. clypeatus*. Flavour production was observed always for *G. chrysimyces* but not at all for *P. papillionaceus* against any micronutrient make up of the synthetic media. Pigmentation of the broth were reddish for *T.clypeatus*, straw yellow (reddish when none were omitted) for *P papillionaceus* and pink for *G. chrysimyces*. The colour of the broths does not markedly get altered by different micronutrient compositions of the media. The effect of micronutrients on the mycelial yields are as follows (Table 3): (*i*) Omission of Cu^{++} is beneficial for the growth of *T. clypeatus* but other ions lower growth in the decreasing order as $Zn^{++}>$, $Mo^{++}>Ca^{++}>$ boric acid $>Fe^{++}$. (*ii*) Omission of all the ions were beneficial for the growth of *P. papillionaceus* and their stinulation response upon growth are in the decreasing order as boric acid $>Ca^{++}>Fe^{++}>Mo^{++}>Cu^{++}>Mn^{++}>Zn^{++}$. (*iii*) Omission of Mo^{++} is beneficial for the growth of *G. chrysimyces* but of other ions lower growth in the decreasing order as $Cu^{++}>$ boric acid $>Zn^{++}>Fe^{++}>Mn^{++}>Ca^{++}$.

TABLE 3. EFFECT OF OMISSIONS OF MICRONUTRIENTS

Micronutrients omitted	*T. clypeatus* dry wt. (g/100 ml)	*P. papillionaceus* dry wt. (g/100 ml)	*G. chrysimyces* dry wt. (g/100 ml)
Boric acid	2.774	2.779	0.514
$CuSO_4$, $5H_2O$	3.344	2.384	0.336
$FeSO_4$, $7H_2O$	2.904	2.472	0.609
$MnCl_2$, $4H_2O$	2.626	2.144	0.690
$MaMoO_4$, $4H_2O$	2.425	2.426	0.882
$ZnSO_4$, $7H_2O$	2.148	2.112	0.582
$CaCl_2$, $2H_2O$	2.433	2.712	0.811
None	3.013	1.408	0.812
All	0.910	2.958	0.414

Mycelial yields at various initial pH values: The growth of mushrooms at various initial pH values and corresponding changes in final pH values of the fermented broths are presented in Fig 4,5 and 6. *T. clypeatus* and *P. papillionaceus* are very similar in their behaviour, both having pH optima 2.5 and their buffring capacity are similar with respect to variation of initial pH of the medium. But *G. chrysimyces* has pH optima at 5.0 and buffering capacity is also different. The buffering capacities of *T. clypeatus* and *P. papillionaceus* are predominant at lower pH values upto 6 and less marked at higher values. On the other hand, *G. chrysimyces* was found to have lower capacity at low pH values but higher in higher pH range.

Growth curves and variation of pH of the fermented broth: Growth curves of these mushrooms, determined in the best selected synthetic media and at optimal pH, are given in Fig. 7,8 and 9. In formulation of these media, concentrations corresponding to higher C/N ratios were taken. The highest mycelial yields per 100 ml and corresponding days required to attain, were for *T. clypeatus*, *P. papillionaceus* and *G. chrysimyces*;

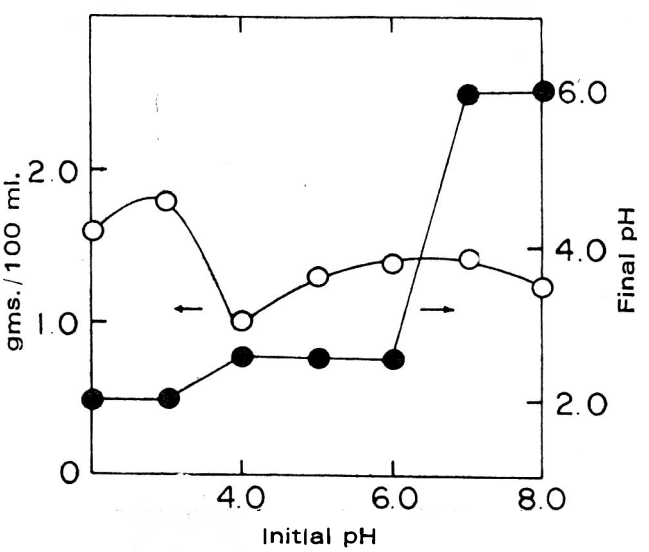


Fig. 5. The influence of initial pH on the mycelial yield of *P. papillionaceus* ●-●-●, final pH; O-O-O-, mycelial dry weight.

TABLE 4. ANALYSES OF THE DRY MYCELIA (H PER 100 G MYCELIA)

Organism	N	Protein (N×6.25)	Carbohydate*	Fat (ether extract)	Fibre	Ash
T. clypeatus	5.08	31.76	52.0	1.0	10.5	2.7
P. papillionaceus	4.804	30.02	34.7	1.23	24.15	5.0
G. chrysimyces	4.356	27.23	49.2	5.15	24.25	0.5

Average of triplicate analysis.
*glucose equivalent.

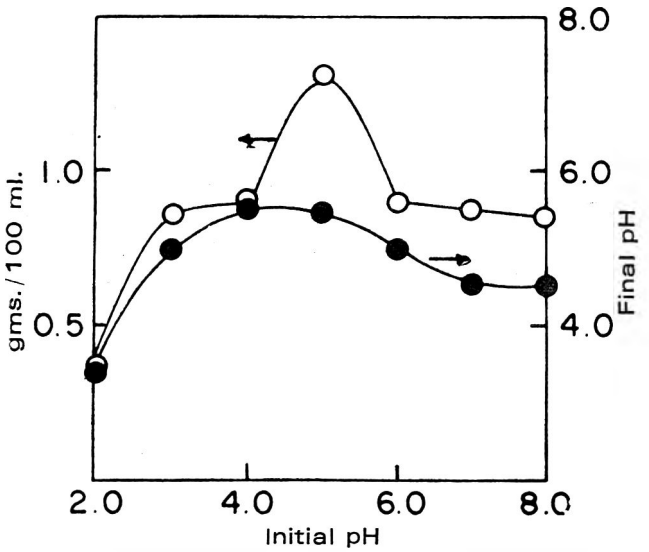


Fig. 6. The influence of initial pH on the mycelial yield of *G. chrysimsces*. ●-●-●, final pH; O-O-O, mycelial dry weight.

2.8 in 18 days; 4.4 g in 14 days and 1.8 g in 10 days respectively. The changes in pH values of the media during the fermentation are also represented in the figures.

Biochemical analyses of mycelial bodies: The analyses of dried mycelial bodies at their growth maxima are given in Table 4. Their protein contents are very similar, but differ in carbohydrate, fat, fibre and ash contents.

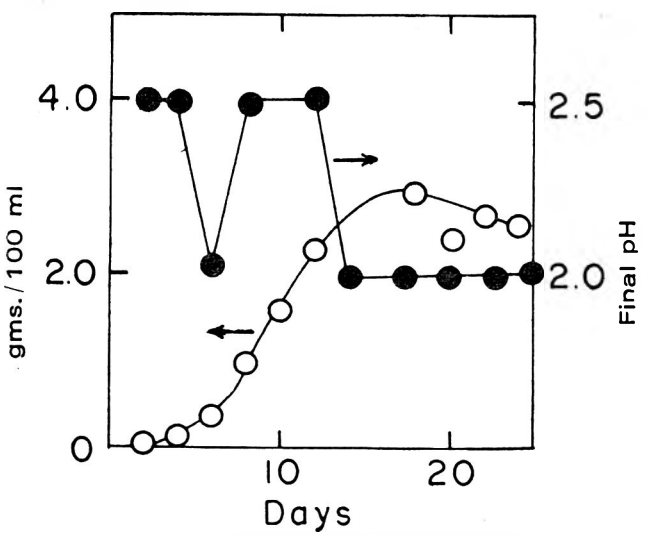


Fig. 7. Growth curve for *T. clypeatus* and the pH variation during fermentation, O-O-O,-mycelial dry weight; ●-●-●,-pH value.

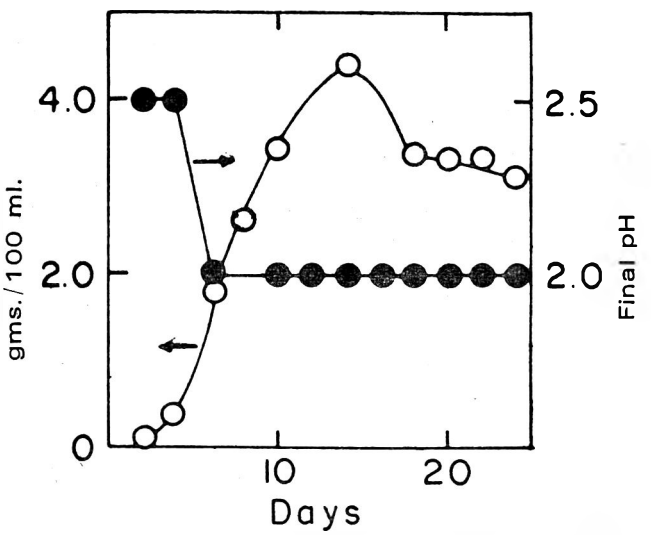


Fig. 8. Grow.h curve for *P. papillonaceus* and the pH variation during fermentation, O-O-O,-mycelial dry weight; ●-●-●,-pH value.

Discussion

The discussion, aimed at the studies of some characteristic features of mushroom fermentation in synthetic medium, reveals many interesting phenomena

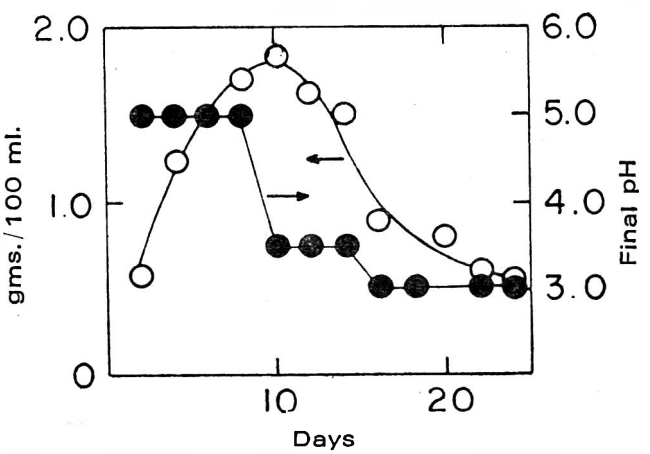


Fig. 9. Growth curve for *G. chrysimyces* and the pH variation during fermentation, O-O-O, mycelial dry weight; ●-●-●, pH value.

associated with propagation of three mushrooms: *T. clypeatus*, *G. chrysimyces* and *P. papillionaceus*. Thus it appears that substitution of glucose by its polymer like dextrin, soluble starch at the same conconcentrations, in common, stimulates growth. It is 10 times for *G. chrysimyces*, 14 times for *P. papillionaceus* and 28 times for *T. clypeatus*. It is also interesting to note that lignin has also some growth supporting activities while glucose is a poor growth supporter. This type of unfavourable effect of glucose on growth has not been found for *A. bisporus*, a much well studied mushroom[15]. It is also observed that for *T. clypeatus* and *P. papillionaceus* malt extract and potato extract of the complex media may be successfully replaced by $NH_4H_2PO_4$, but for *G. chrysimyes* urea does not appear to be equivalent substitute (Table 1 & 2).

The importance of C/N ratio for mushroom growth has been explained by various workers[16,17]. But in the present investigation, it has interestingly been observed of the occurrence of at least two C/N ratios in synthetic media at which favourable growth is attained. One being common at 5 and others are at 10, 15 and 26 respectively for *T. clypeatus*, *G. chrysimyces* and *P. papillionaceus*. Inspite of the variation of higher values of C/N, growth attained at these values are approximately double to those at lower values. Thus it appears that there might be some difference in the nitrogen metabolism of mushroom compared to other microbes.

All the mushrooms under investigation have very low pH optima and always show acid reaction during fermentation.

It has also been observed that micronutrient compositions of the synthetic media not only affect the growth but also flavour production. We have already reported the importance of Ca^{++} concentration of the synthetic medium for the production of flavour in propagation of *V. volvacea*[18]. It has further been noticed here that micronutrients used in this investigation are not at all required for the growth of *P. papillionaceus*, and addition of any of the ions was found unfavourable to its growth in the concentrations tested. But for all the strains, the pure mushroom flavour could not be achieved in synthetic media other than a fruity flavour which was also been reported by other workers[4].

Thus the present investigation highlights some of the characteristic features of mushroom fermentation and also suggests the following synthetic media (composition in g/100 ml of medium);

T. clypeatus: Dextrin-10; $NH_4H_2PO_4$-2.463; boric acid-0.057; KH_2PO_4-0.087; $MgSO_4$, $7H_2O$-0.05; $FeSO_4$, $7H_2O$-0.025; $MnCl_2$, $4H_2O$-0.0036; $NaMoO_4$, $4H_2O$-0.0032; $ZnSO_4$, $7H_2O$-0.03; $CaCl_2$, $2H_2O$-0.037; pH-2.5.

P. papillionaceus: Soluble starch -9; $NH_4H_2PO_4$-2.463; KH_2PO_4-0.087; $MgSO_4$, $7H_2O$-0.05; pH-2.5.

G. chrysimyces: Soluble starch-5; Urea-0.214; KH_2PO_4-0.087; $MgSO_4$, $7H_20$-0.05; $CuSO_4$, $5H_2O$-0.0039; $FeSO_4$, $7H_2O$-0.025; $MnCl_2$, $4H_2O$-0.0036; $ZnSO_4$, $7H_2O$-0.03; $CaCl_2$,$2H_2$ 0-0.037; boric acid-0.057; pH-5.0.

Acknowledgement

The kind cooperation of Dr. N. Samajpati, department of Botany, Calcutta University, is gratefully acknowledged for the identification of the mushroom strains. Authors are grateful to the Council of Scientific and Industrial Research, New Delhi for providing a fellowship to one of them (A.K.G.) and to Indian National Science Academy, for contingency help to S.S.G. Acknowledgements are due to Prof. B. K. Bachhawat, Director, Indian Institute of Experimental Medicine for his kind interest in the work. Technical assistance of Sri Pranabendu Majumder, is also acknowledged.

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Xylanase Production by Ultra Violet Induced Variants of *Streptomyces fradiae* SCF-5

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Attempts were made to improve the xylan degrading ability of *S. fradiae* SCF-5 by induced mutation with ultra-violet light. Variants having nearly two-fold increase in xylanase activity were isolated. The variation appeared to be not only with respect to the increased level of xylanase activity, but also in the types of xylanases produced in the variants. High xylanase yielding variants could grow on natural xylan substrates producing extracellular xylanase but with little detectable cell bound glucose-isomerase.

Glucose-isomerase used in the preparation of high fructose syrups is known to be produced by many microorganisms as an adaptive enzyme in presence of xylose in the medium[1]. In very few cases as far as we know, it has been reported that streptomyces strains possessed both glucose-isomerase and xylan degrading enzymes by virtue of which they could be cultivated on natural xylan substrates[2,3]. We have screened, unsuccessfully, a number of streptomyces for the presence of both xylan degrading enzyme and glucose-isomerase in the same culture[4]. One of the potent glucose-isomerase producers viz. *S. fradiae* SCF-5 isolated earlier in this laboratory[5], on examination, revealed the presence of small extent of xylan degrading property in its culture fluid. In the present work we have made attempts to improve the xylan degrading ability of this organism by mutation with ultra violet light and examine the possibility of cultivating it on natural xylans for the production of glucose-isomerase.

Materials and Methods

S. fradiae SCF-5 was maintained on Bennett Agar Slants at 4°C and transferred fortnightly.

The basal medium (I) employed for xylanase production was of the following composition: $(NH_4)_2SO_4$, 0.26 per cent; K_2HPO_4, 0.24 per cent; KH_2PO_4, 0.57 per cent; yeast extract, 0.05 per cent; $MgSO_4$, 0.1 per cent and a carbon source, 1.0 per cent, pH 7.0. For glucose-isomerase production, the above basal medium was supplemented with 0.024 per cent $CoCl_2$ (II).

Fifty ml of the medium in a 250 ml conical flask was inoculated with a loopful of streptomyces spores and cultivated at room temperature in gyrotary shaker for 48-72 hr for the production of enzymes. The assay procedure for glucose-isomerase and definition of its unit have already been given[5]. Xylanase was assayed in 2 ml of reaction mixture containing larch wood xylan (1 per cent), 0.5 ml; sodium phosphate buffer (0.05 M), 0.5 ml; enzyme, 0.1 ml and distilled water 0.4 ml; incubated for 30 min. The reducing sugars formed were estimated by using the 3,5 dinitrosalicylic acid reagent[6]. Since we were dealing with a new system, the pH and temperature optima for the xylanase activity in culture fluid were determined (Fig. 1). They were respectively pH 6.0 and 55°C and all xylanase assays were done under these optimum conditions. One unit of xylanase was defined as that amount of enzyme which gave rise to the formation of 1 μ mole of xylose per min under the assay conditions.

UV irradiation: Spores from a 4-day old slant culture or fragmented vegetative cells from the early stationary phase of growth taken in physiological saline containing 0.05 per cent Tween-80 were subjected to UV irradiation at a distance of 29 cm from a Hanovia model—UV lamp. Usually 10 ml suspension containing 10^8 cells ml^{-1} was subjected to irradiation with constant agitation in an open 10 cm. diameter petridish. Irradiated culture was refrigerated overnight prior to plating out for viable count and assessment of mutagenesis.

For observing xylanase producing variants the irradiated culture was plated out on basal medium agar (I) containing 0.02 per cent xylan which imparted turbidity to the medium. Clearance zone around a colony indicated degradation of xylan.

A summarised version of this paper was presented at the First Indian Convention of Food Scientists and Technologists, 23-24, June 1978, at C.F.T.R.I., Mysore-570 013.

Chromatographic analysis of xylan degradation products: Twenty ml of the reaction mixture containing xylan (Sigma) 1 per cent, 4 ml; PO_4 buffer (0.05 M, pH 6.0), 4 ml and culture fluid (as source of xylanase), 4 ml was incubated at 55°C overnight. The sugars formed were analysed by paper chromatography in ethyl acetate, pyridine, water (8:2:1) and butanol, acetic acid, water (4:1:1) systems by ascending technique using Whatman No. 3, chromatography paper and visualised by spraying with $AgNO_3$[7] or Benzidine[8] reagents.

Larchwood xylan was purchased from Sigma Chem. Co., U.S.A. and D-xylose from B.D.H., England. The other reagents were of analytical grade, procured within the country.

Results and Discussion

Effect of ultra violet (UV) irradiation on survival and mutagenesis in S. fradiae: Both spores and vegetative cells, in separate trials, were subjected to UV irradiation to assess the suitability of the test material. Spores often remained as clumps and were floating on the suspension fluid. A fairly uniform suspension of spores could be obtained in saline containing 0.05 per cent Tween-80.

Usually at the beginning of stationary phase of growth, *S. fradiae* mycelia were found to undergo fragmentation into short rods resembling any typical rod shaped bacterium. A good uniform cell suspension could be made from the culture at this stage of growth. Sometimes a tissue homogeniser was used to make uniform spores or vegetative cell suspension.

In Table 1 are given the survival pattern of the culture after subjecting the spores and vegetative cells to different periods of exposure to UV light. Surprisingly, in a number of trials, we have found that the vegetative cells were more resistant to UV kill than the spores at lower dosage of UV irradiation. At higher dosage of UV, however, the vegetative cells appeared to be inactivated at a faster rate than the spores. The possible explanation for the above observation is that vegetative cells, being actively metabolishing would be able to repair the damages caused by lower dosages of UV and hence are more resistant than the dormant spores.

TABLE 1. EFFECT OF UV EXPOSURE PERIOD ON SURVIVAL OF *S. FRADIAE* SCF-5

Exposure period (sec.)	Survival (%) after exposure of Vegetative cells	Spores
0	100	100
20	23.20	12.60
40	0.92	2.60
60	0.07	0.28
120	0.00	0.07

Various kinds of morphological variants were discerned on the plates. More commonly we obsrved the nonsporulating or bald variants. There were colonies which appeared whitish or greyish in comparison to the greyish pink parental culture.

On the xylan agar plates (medium I), the colonies of the parental cultures also produced a clearance zone of about 5 mm diameter 48 hr after plating. Any colony which showed considerably bigger clearance zones than the parental colonies were picked up as xylanase variants and subjected to further test for the production of enzymes in the fluid basal medium I containing 1.0 per cent wheat bran and 0.1 per cent glucose. The frequencies of various levels of xylanase producers from the irradiated and the control unirradiated samples selected by the above procedure have been given in Fig. 2. It was clear that the irradiated cultures exhibited a definite increase in production of extracellular xylanase.

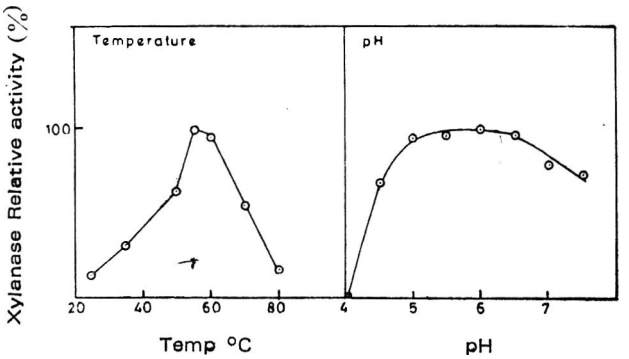

Fig. 1. Temperature and pH effect on xylanase activity of the culture fluid of *Streptomyces fradiae* SCF-5

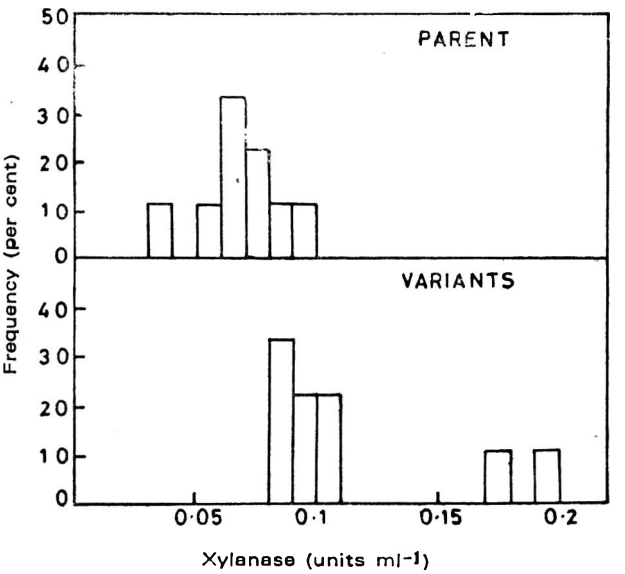

Fig. 2. Xylanase production in parent and variants of *Streptomyces fradiae* SCF-5

While most of the parental colonies possessed xylanase ranging from 0.05 to 0.06 units ml^{-1}, the irradiated cultures had yielded colonies producing 0.19 to 0.2 units ml^{-1}. Three of the high xylanase yielding variants viz. UV, UV_2 and Grey were selected for further studies.

Hydrolysis of xylan by the culture fluids of UV_1, UV_2 and Grey: The data on chromatographic analysis of sugars formed by hydrolysis of xylan by the culture fluids of above variants of SCF-5 over a period of 4 to 72 hr have been given in Table 2. In the early stages, i.e. from 4 to 24 hr, spots having lower mobility than xylose were detected on the chromatogram. The spot-co-chromatographing with authentic D-xylose was found only in case of samples incubated for 72 hr. While in the case of variant UV-1 the xylose spot and other low mobility spots were still found after 72 hr. incubation, in the variant UV-2 xylose spot, and another spot with higher mobility than xylose were seen. With the Grey variant the xylose spot was not detected, although the number of low mobility spots decreased from 4 during the early period of incubation to just 1 after 8 hr. The above results perhaps suggest that UV has induced variations of xylanase production in this culture not only quantitively but also with respect to the quality or type of the xylanase formed. The exact manner in which this qualitative change has occurred is yet to be studied. We presume at the moment that the parental culture produces a mixture of xylanases among which one or more types are promoted or magnified in the xylanase variants. Different kinds of xylanases, with regard to their mode of action on the xylan molecule are known[9] according to which the products of xylan hydrolysis would be xylose and/or xylo-oligo-saccharides.

TABLE 2. HYDROLYSIS OF XYLAN BY CULTURE FLUIDS OF *S. FRADIAE* VARIANTS

S. *fradiae* SCF-5 variant	Hydrolysis period (hr)	Sugar spots on paper chromatogram*					
		1	2 (xylose)	3	4	5	6
UV_1	4	—	—	+	+	—	—
	8	—	—	+	+	—	—
	24	—	—	+	+	—	—
	72	—	+	+	+	—	—
UV_2	4	—	—	+	+		—
	4	—	—	+	+	+	+
	8	—	—	+	+	+	—
	24	—	—	+	+	—	—
	72	+	+	—	—	—	—
Grey	4	—	—	+	+	+	+
	8	—	—	+	—	—	—
	24	—	—	+	—	—	—

*Numbering with decreasing order of mobility.
–Spot absent; +Spot present.

TABLE 3. XYLANASE PRODUCTION BY *S. FRADIAE* UV_2 VARIANT AFTER GROWTH ON VARIOUS SUBSTRATES

Substrate[a]		Xylanase (units ml^{-1})
Wheat bran		0.057
Wheat bran residue[1]		0.101
do	(preincubated)[b]	0.155
Paddy straw		Nil
Paddy straw residue[2]		0.101
do	(preincubated[b])	0.110
Saw dust		Nil
Bagasse		Nil

[a]Substrate at 1.0 per cent level.
[b]Incubation done at 55°C for 24 hrs.
[1]Wheat bran residue obtained after cultivation of *Aspergillus carbonarius* for pectinase production.
[2]Paddy straw residue after cultivation of *Pleurotus flabullatus*.

Growth of UV_1, UV_2 and Grey on natural xylan substrates and enzyme induction: Attempts were made to cultivate the above xylanase variants in the basal medium (I) containing various natural xylan substrates such as wheat bran, saw dust, paddy straw, bagasse, etc. In the large scale production of pectinase by *Aspergillus carbonarius* in tray fermentation, a considerable quantity of wheat bran residue is discarded; likewise the paddy straw residue is a waste product after mushrooms have been raised on them. These two waste materials were also tested in the present work as growth substrates.

In most media, considerable levels of xylanase were produced extracellularly by the *S. fradiae* variants, but cell bound glucose-isomerase was found only in traces in some media and was completely undetectable in others. The variants were rechecked for their ability to produce glucose-isomerase in presence of xylose and they were found to be capable of producing the enzyme. The variants produced more xylanase in wheat bran or paddy straw residue than in the respective fresh unused substrates (Table 3). In the preincubated residues kept at 55°C for 24 hr, xylanase production was still better. It may therefore, be inferred that some predigestion of the xylans is necessary and that the residues probably possess enzymes elaborated by the fungi or mushroom and during preincubation, the xylans in them are further broken down. This probably would mean that although the *S. fradiae* variants were capable of improved production of xylanase, the enzymes in them were either

insufficient, or inefficient to generate sufficient xylose for induction of glucose-isomerase.

Acknowledgement

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Stimulation of Glucose-Isomerase Production in *Streptomyces fradiae* SCF-5 by Enzyme Hydrolyzed Wheat Bran

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Wheat bran was partially hydrolyzed with crude streptomyces xylanase. This hydrolyzate promoted glucose-isomerase production in *S. fradiae* SCF-5 at levels comparable to those obtained with xylose. The wheat bran hydrolyzate was fractionated by gel filtration and one of the fractions which promoted high production of glucose-isomerase contained an oligosaccharide rather than xylose.

Glucose-isomerase is now one of the important commercial enzymes used in the production of high fructose syrups. A large proportion of microorganisms reported as producers of glucose-isomerase need xylose as inducer for the enzyme biosynthesis. For economic production of enzyme, natural xylan Containing substrates such as wheat bran, corn cobs, etc. could be utilised but organisms possessing both glucose-isomerase and xylan degrading ability were found only in a few instances[1,2]. In the present work, therefore, we have tested the possibility of employing two Streptomyces cultures, one possessing a powerful xylanase activity (but itself lacking in glucose-isomerase) and the other capable of producing high levels of glucose-isomerase adaptively in presence of xylose in the growth medium. Either the two cultures were grown together on wheat bran or wheat bran hydrolysed with the culture fluid of the first organism was used as inducer in the second, in the ultimate production of high levels of glucose-isomerase by the second organism in either procedure.

Materials and Methods

Isolation and maintenance of streptomyces cultures: Streptomyces were isolated from locations rich in natural xylans according to procedures already described[3]. *S. fradiae* which has been described earlier[3,4] was employed as the producer of glucose-isomerase. The cultures were maintained on Bennett agar slants and wheat bran agar slants (yeast extract, 0.25; meat extract, 0.25; $MgSO_4$, 0.05; corn steep liquor, 0.5; groundnut meal, 0.5; wheat bran, 1.0 and agar, 2.0 per cent at pH 7.0).

Media and conditions for enzyme production: Xylanase production was carried out by submerged fermentation by inoculating one loopfull of spores to 50 ml of wheat bran medium of above composition in a

A summarised version of this paper was presented at the First Indian Convention of Food Scientists and Technologists, 23-24, June 1978, at C.F.T.R.I., Mysore-570 013.

250 ml capacity conical flask and cultivating at room temperature (25-30°C) for 3-4 days in a gyrotary shaker. For production of glucose-isomerase wheat bran medium containing 0.024 per cent $CoCl_2$ and peptone medium (peptone, 1.0; meat extract, 0.5; yeast extract, 0.25; NaCl, 0.5; $CoCl_2$, 0.024; $MgSO_4$, 0.05; glucose, 0.3 and xylose, 0.5 per cent at pH 7.0) were employed.

The procedures of glucose-isomerase and xylanase assays and definition of enzyme units have already been given[3,5].

Preparation of wheat bran hydrolysate: Fifty grams of wheat bran was suspended in 100 ml of distilled water. To this 125 ml of xylanase enzyme solution (culture fluid) was added and incubated at 55°C overnight. The hydrolysed material was filtered through a cheese cloth.

Total sugar content of the wheat bran hydrolysate was determined using the Anthrone method[6]. Pentose was estimated using phloroglucinol reagent[7].

Fractionation of wheat bran hydrolysate was done with Sephadex G-25 (fine) column of size 40×4.5 cm. The column was charged with 160 mg of total sugar in 1.5 ml solution, and eluted with distilled water at a flow rate of 15 ml per hr. 2.5 ml fractions were collected. Sugars were analysed by paper chromatography in the solvent system of butanol: acetic acid: water (4:1:1) by the ascending technique using Whatman No. 3 chromatography paper. The sugar spots were visualised by spraying the paper with Benzedine reagent[8].

TABLE 1. PRODUCTION OF XYLANASE AND GLUCOSE-ISOMERASE BY DIFFERENT ISOLATES OF STREPTOMYCES

Streptomyces isolates	Xylanase (Units/ml)	Glucose-isomerase (Unitsml^{-1})
ISS_1K	18.20	0.6
CFT_3	16.86	1.0
MFS_1	1.93	0.1
MC_36	10.00	0.1
4b	2.20	0.2
MC_12_2	14.00	1.6
3b	0	0.2
CFT_{12}	10.20	0.8
MC_68	1.77	0.2
MC_1	30.06	1.6
HM_4	4.00	0
CFT_{14}	6.20	0.4
MC_121	13.33	0
3a	16.40	0
CFT_1	11.53	0
CFT_{10}	9.33	0.4
MC_64	4.00	0.2

TABLE 2. EFFECT OF MIXED CULTIVATION OF XYLANASE PRODUCING STREPTOMYCES AND *S. FRADIAE* SCF-5 ON GLUCOSE-ISOMERASE PRODUCTION

Xylanase culture in the medium	Glucose-isomerase (Unitsml^{-1})
ISS_1K	0.2
CFT_{12}	0.8
MC_121	1.0
MC_1	1.6
3a	0.4

Results and Discussion

Testing cultures for the simultaneous presence of xylanase and glucose-isomerase: A number of streptomyces cultures were examined for the presence of xylanase in the culture fluid and glucose-isomerase in the cells after the growth of the culture in the wheat bran medium for 72 hr. The results given in Table 1 indicate that good xylanse producers do not possess corresponding levels of glucose-isomerase. From among these culture isolates, MC_1, ISS_1K, 3a, MCI-21 and CFT_{12} were selected as good xylanase producers.

Effect of mixed cultivation of xylanase and glucose-isomerase producing cultures on the production of glucose-isomerase: The glucose-isomerase producing culture *S. fradiae* SCF-5 was cultivated along with each of the five potent xylanase producing streptomyces in wheat bran medium, in order to test whether the xylanase cultures would make available sufficient xylose by hydrolysis of wheat bran for induction of glucose-isomerase in *S. fradiae* SCF-5. It would be seen as per the results given in Table 2 that no pronounced effect on glucose-isomerase production by SCF-5 was evident. This could probably be due to competition of the cultures for the growth substrates.

Effect of sequential cultivation of xylanase and glucose-isomerase producing cultures: In this experiment the xylanase producing culture was grown first for 72 hr in the wheat bran medium and then the whole medium along with the culture was sterilized by autoclaving. This medium was inoculated with *S. fradiae* SCF-5 and

TABLE 3. EFFECT OF SEQUENTIAL CULTIVATION OF XYLANASE PRODUCING STREPTOMYCES AND *S. FRADIAE* SCF-5 ON GLUCOSE-ISOMERASE PRODUCTION

First organism in the medium	Glucose-isomerase (Unitsml^{-1})
ISS_1K	0
CFT_{12}	0.48
MC_1	0.20
3a	0.40

TABLE 4. EFFECT OF WHEAT BRAN HYDROLYSATE, HYDROLYSED WITH THE CULTURE FLUIDS OF DIFFERENT XYLANASE PRODUCING STREPTOMYCES ON GLUCOSE-ISOMERASE PRODUCTION BY *S. FRADIAE* SCF-5

Culture fluid of the organism employed in wheat bran hydrolysis	Glucose-isomerase (Units ml^{-1})
ISS_1K	5.0
CFT_{12}	2.0
MC_121	2.4
MC_1	4.8
3a	2.0
$CFT_{12}+MC_121$	2.8
MC_1+MC_121	1.4
MC_1+CFT_{12}	1.4
MC_1+ISS_1K	1.6

cultivation continued for 48 hr at the end of which cells were harvested and assayed for glucose-isomerase. The results given in Table 3 indicate that here also the production of glucose-isomerase was rather poor presumably due to depletion of nutritents in the medium by the microorganism cultivated first.

Induction of glucose-isomerase with enzyme hydrolysed wheat bran: From the foregoing it is clear that two microorganisms among the cultures tested in this work, one producing xylanase and the other glucose-isomerase would not function cooperatively for the production of glucose-isomerase by their growth on a natural xylan containing substrate like the wheat bran. Therefore, attempts were made to hydrolyse wheat bran with the culture fluid of a xylanase producing streptomyces and employ this hydrolysate as the substrate for the production of glucose-isomerase by the second microorganism. Table 4 gives the results obtained on the production of glucose-isomerase by *S. fradiae* SCF-5 after growth in peptone medium containing wheat bran hydrolysate (10 per cent V/V); hydrolysed with culture fluids of different streptomyces. In the case of two samples one hydrolysed with culture fluids of streptomyces isolate ISS_1K and the other with that of MC_1 high yields of glucose-isomerase viz. 5 and 4.8 (units ml^{-1}) respectively were obtained. In some samples, where mixtures of culture fluids were employed for hydrolysis of wheat bran, however, the glucose-isomerase production was rather poor. This was surprising in view of our earlier finding that in some combinations of culture fluids synergistic effect, as high as 200 per cent increase, in the hydrolysis of xylan was noticed[9]. One possible explanation for the low production of glucose-isomerase with mixtures of xylanase culture fluids could be that extensive hydrolysis of wheat bran is not the ideal condition for induction of glucose-isomerase in producing streptomyces. Data presented in Table 5 further substantiate this hypothesis. In this experiment a comparison has been made between xylose and wheat bran hydrolysate on their ability to induce glucose-isomerase in *S. fradiae* SCF-5. We found, for the same pentose content there was a higher extent of enzyme induction by wheat bran hydrolysate than by xylose. At 0.16 per cent pentose content xylose solution was able to induce only 1.64 units of glucose-isomerase/ml while for the same concentration of pentose, the wheat bran hydrolysate induced 5 units of enzyme/ml.

If extensive wheat bran hydrolysis was not ideal then the partial hydrolysis may be desirable for two reasons: (1) extensive hydrolysis would probably generate other sugars inhibitory to glucose-isomerase formation or (2) an oligosaccharide/oligossaccharides from the partially hydrolysed wheat bran would be a better inducer of glucose-isomerase than xylose itself. Some evidence for the second possibility was obtained when we fractionated

TABLE 5. RELATIVE EFFICIENCY OF XYLOSE AND WHEAT BRAN HYDROLYSATE ON THE PRODUCTION OF GLUCOSE-ISOMERASE IN *S. FRADIAE* SCF-5

Pentose %	Glucose-isomerase (Units ml^{-1}) induced with	
	Xylose	Wheat bran hydrolysate
0.04	0.54	0.90
0.08	1.04	1.32
0.12	1.22	1.74
0.16	1.64	5.00
0.24	1.22	2.40
0.32	2.12	1.34
0.48	2.60	0.38

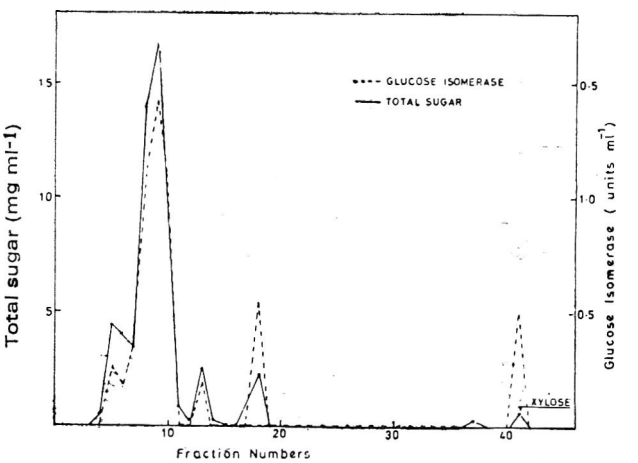

Fig. 1. Fractionation of wheat bran hydrolysate on Sephadex G-25 and induction of glucose-isomerase in *Streptomyces fradiae* SCF-5 by different fractions.

the wheat bran hydrolysate on Sephadex (G-25) column and one of the fractions which gave the highest induction of glucose-isomerase/unit total sugar used was found to be not xylose. This substance which showed much lower mobility on the paper chromatogram was presumably an oligosaccharide (Fig. 1).

From the above data we may conclude that an inexpensive inducer such as enzyme hydrolysed wheat bran can be employed for economic production of glucose-isomerase, and that an oligosaccharide (containing xylose?) is a better inducer than xylose itself.

Acknowledgement

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Single Cell Protein Production from Corn Cobs

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The chemical composition of corn cobs is given. The corn cobs hydrolysate contained glucose, mannose, xylose, arabinose and uronic acids. Of the fifteen cellulolytic fungi grown on corn cobs, *M. verrucaria* showed maximum protein production (204.6 mg) in the biomass. Of the seven nitrogen sources (250 to 600 mg N/l) only potassium nitrate at a level of 350 to 400 mg N/l was found suitable. The five different phosphorus sources tried did not show significant effect on SCP production. The optimum pH range 5.0 to 5.5 was observed for SCP. The highest concentration of CMase activity was shown within 48 to 72 hr after inoculation. The maximum CMase activity was shown by *M. verrucaria* (4.42 units/ml) upto 72 hr after inoculation.

Chahal and Gray[1] and Singh *et al*[2] produced fungal protein by growing cellulolytic fungi on wood pulp and wheat straw, respectively. Moo Young and Chahal[3] converted various insoluble cellulose materials in SCP by employing *Chaetomium cellulolyticum*. Mills[4] reported bio-deterioration of cellulose by thermophilic fungi. Ek and Eriksson[5] converted lignocellulosic waste into protein by a fungus (*Sporotrichum pulverulentum*).

In the present study an attempt has been made to utilize cellulosic waste material such as corn cobs for protein production.

Materials and Methods

Corn cobs were chopped and dried in hot air oven at 60°C, ground in Wiley Mill, passed through 60 mesh sieve and stored for analysis and SCP production.

Chemical Analysis: Ether extract, crude fibre, moisture and ash were determined by the methods of AOAC[6]. The holocellulose and hemicellulose were also determined[7]. Crude protein[8] (N×6.25), lignin[9] and reducing sugars were estimated[10].

Paper chromatography of hemicellulose: The hydrolysis of hemicellulose was done by the technique of Myhre and Smith[7]. Throughout this study, descending paper partition chromatography for qualitative analysis of sugars and sugar-acids was employed. The solvent system of n-butanol: acetic acid: water (4:1:5 v/v) of Partridge was used[11]. The spray reagent benzidine trichloroacetic acid[12] and resorcinol[13] were employed.

Production of single cell protein(SCP): The ground corn cobs were partially delignified[14]. Half gram samples of delignified corn cobs were taken in 250 ml Erlenmeyer

flask and 50 ml of Chahal and Gray[15] medium, devoid of any carbon source, was added and pH adjusted to 5.5. Fifteen fungi used *Myrothecium verrucaria*, *Cochliobolus* sp., *Chaetomiumg lobosum*, *Fusarium* sp. I., *Fusarium* sp. II, *Aspergillus niger* I, *Aspergillus niger* II, *Asp. terreus*, *Penicillium* sp., *Alternaria tenis*, *Alternaria* sp., *Alternaria alternaria*, *Pen. funiculosum*, *Rhizopus* sp. and *Mucor* sp. were obtained from the culture collections of the Department of Microbiology. Cultures were incubated as shake culture for 8 days at 28±0.5°C. The biomass was harvested and dried at 40°C under vacuum.

Cellulase activity: Cellulase activity (CX) in the culture filtrate was determined by the method of Miller *et al*[16].

Nitrogen source: Seven different nitrogen sources, potassium nitrate, sodium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, diammonium hydrogen phosphate and urea were employed in concentration of 250 to 600 mg of N/l of medium.

Phosphorus source: Five phosphorus sources i.e., potassium dihydrogen phosphate, dipotassium hydrogen phosphate, diammonium hydrogen phosphate, disodium hydrogen phosphate and phosphoric acid were added in concentrations of 400 to 800 mg P/l of medium.

Results and Discussion

The cellulose, hemicellulose, soluble sugars, lignin, crude protein, crude fat and ash contents of corn cobs are given in Table 1. Sugars in the corn cobs hydrolysate comprised of glucose, mannose, xylose, arabinose and uronic acids.

The protein production by fifteen fungi ranged from 10.2 to 28.12 per cent and *Myrothecium verrucaria* gave the highest percentage of crude protein (28.12 per cent). Total crude protein in the biomass ranged from 70.8 to 204.6 mg/g of the substrate and highest amount of total protein (204.6 mg) was shown by the same organism. (Table 2.)

Final pH of cell free filtrate ranged from 5.2 to 6.2. A pH of 5.2 was recorded by *Aspergillus niger*. But, this value is much higher than that reported by Reusser *et al*[17]. It might either be due to strain variation or different substrate employed in the present study. The highest (3.7 units) and lowest (1.0 units) CMase activity was observed in the culture filtrate of *M. verrucaria* and *P. funiculosum*. respectively. Reducing sugars content ranged from 0.404 to 2.146 mg/ml of filtrate.

Effect of different nitrogen (N) sources on SCP production was tried in five fungal cultures (Fig. 1). Potassium nitrate supported maximum SCP by *M. verrucaria; Cochliobolus* sp. *C. globosum* and *Rhizopus* sp., while urea proved to be the best for *Asp. terreus*. Ammonium dihydrogen phosphate was comparable to potassium nitrate as N source, when protein production by *M. verrucaria* was considered.

Each N. source was tried at 7 different levels ranging from 250 to 600 mg N/l. The optimum concentration of N for SCP production is dependent upon the type of fungus used. Four hundred mg N was found to be optimum for *M. verrucaria; Cochliobolus* sp; *Alternaria* sp. as potassium nitrate N; *M. verrucaria*, *Cochliobolus* sp. *C. globosum* as sodium ntirate N; and *M. verrucaria*, *C. globosum* and *Rhizopus* sp. as diammonium hydrogen phosphate N.

On the other hand 350 mg N was optimum for *C. globosum*, *Rhizopus* sp. as potassium nitrate N; *A. terreus*, *Rhizopus* sp. as sodium nitrate N; and *Cochliobolus* sp. and *A. terreus* as diammonium hydrogen phosphate N. Higher concentrations of N had inhibitory effect upon SCP production; this might be due to change in osmotic pressure leading to higher solute molecular concentration outside the fungal cell. However, the toxic effect of higher concentrations of urea was mainly due to accumulation of NH_4^+ ions released by the breakdown

TABLE 1. CHEMICAL COMPOSITION OF CORN COBS

	(%)
Water soluble sugars	2.70
Cellulose	34.10
Hemicellulose	42.50
Crude fat	0.96
Crude protein	1.64
Lignin	12.80
Total mineral content	5.46

TABLE 2. SINGLE CELL PROTEIN PRODUCTION BY VARIOUS FUNGI

Culture	Final pH	CMase activity	Protein %	Total protein/g of substrate	Soluble sugars mg/ml
M. Verrucaria	6.2	3.7	28.1	204.6	0.762
Cochliobolus sp.	5.9	2.2	22.1	166.9	0.484
C. globosum	6.0	2.5	19.2	119.2	1.763
Fusarium sp. I	5.9	1.9	16.1	104.2	1.260
Fusarium sp. II	5.7	1.5	17.9	105.0	0.984
Asp. niger I	5.2	3.2	13.2	95.3	0.810
Asp. niger II	5.3	2.7	12.1	91.8	1.106
Asp. terreus	5.9	2.9	12.2	84.2	1.815
Penicillium sp.	5.8	2.1	14.3	99.9	0.644
Alternaria tenis	6.1	2.1	15.8	126.6	2.146
Alt. alternaria	6.0	2.2	13.1	97.1	1.337
Alternaria sp.	6.0	1.9	12.7	99.6	0.906
Pen. funiculosum	6.1	1.0	10.2	70.8	1.010
Rhizopus sp.	6.0	2.2	15.1	117.3	1.516
Mucor sp.	5.9	1.8	14.6	99.6	0.784

Average of three trials.

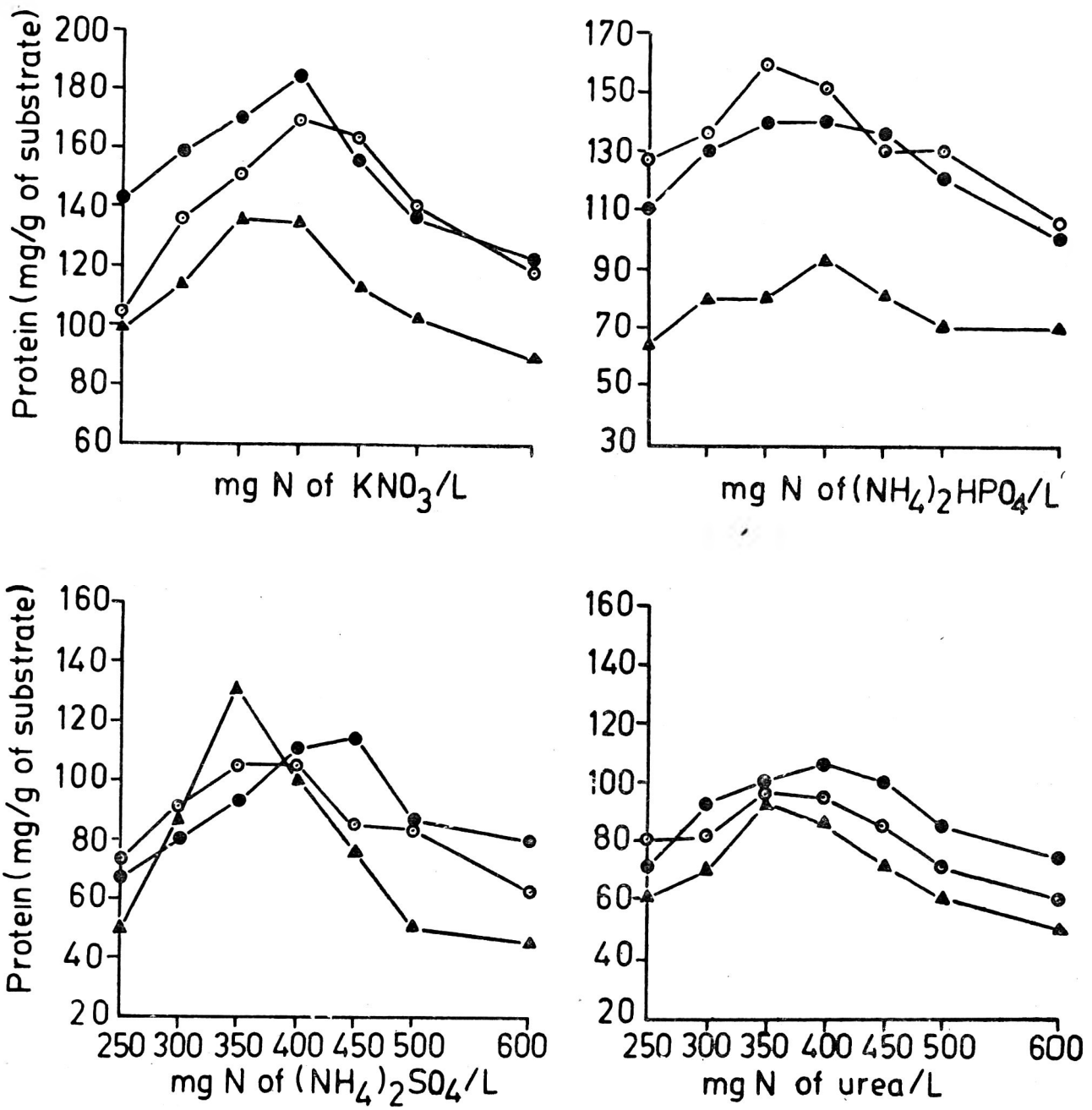

Fig. 1. Effect of different nitrogen sources on protein production

M. verrucaria ●——●
Cochliobolus sp. O——O
C. globosum △——△

of urea by fungal urease. Shukla and Datta[18] recorded similar observations while employing urea as N and molasses as carbon sources. In the present study, ammonia N did not support well the protein production as compared to the nitrate N. However, these findings were corroborated by Chahal and Dhaliwal[19].

In this study, the optimum concentration of potassium nitrate was found to be 400 mg N; beyond this concentration SCP production was inhibited. However, Chahal and Cheema[20] reported inhibitory effect of potassium nitrate above 600 mg N on SCP production by *Penicillium crustosum*. This indicated that *P. crustosum* could withstand higher osmotic pressure of potassium nitrate solutes as compared to fungal species used in this study. Different sources of phosphorus did not show significant effect on protein production by different fungi.

The optimum pH range for SCP production for various fungi was found to be between pH 5.0 and 5.5 (Fig. 2).

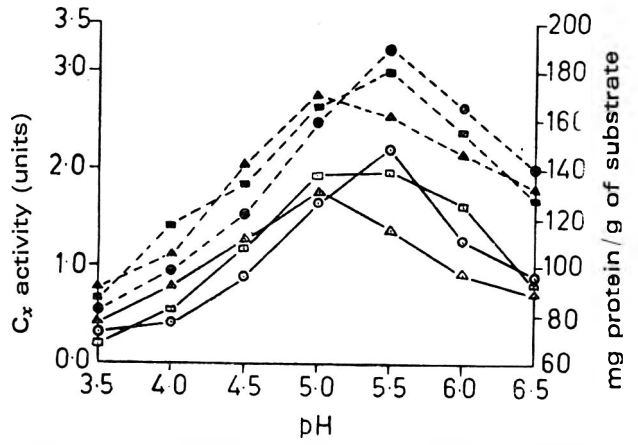

Fig. 2. Effect of pH on C_x activity and protein production

C_x

M. verrucaria O —— O; *Cochliobolus* sp. □ —— □;
C. globosum △ —— △

Protein

M. verrucaria ●——● ; *Cochilobolus* sp. ■——■ ;
C. globosum ▲——▲

The pH optima for protein production by *M. verrucaria*, *Cochliobolus* sp., *A. terreus* and *Rhizopus* sp. was 5.5 while for *C. globosum* it was 5.0 for SCP production.

Cellulase (CMase CX) production by these fungi over a period of 192 hr of incubation is shown in Fig. 3. There was initial lag period for 12 to 24 hr in cellulase

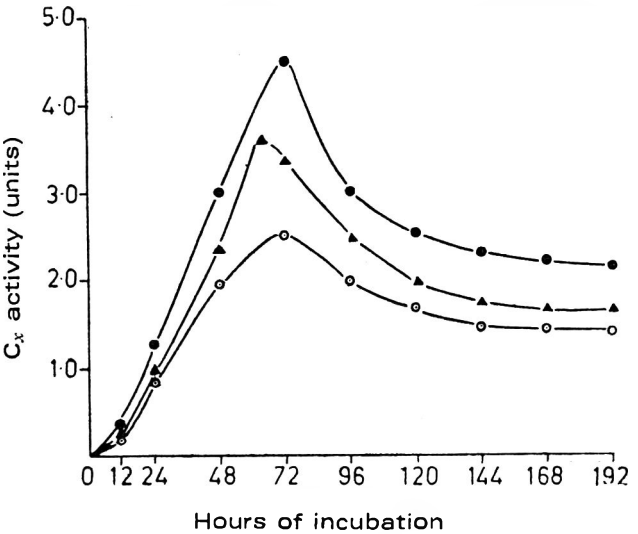

Fig. 3. Effect of incubation period on C_x activity.
M. verrucaria ●—— ●; *Cochilobolus* sp. O —— O;
C. globosum ▲——▲

production, but the concentration reached its maximum within 48 to 72 hr. On further incubation of culture upto 192 hr, enzyme concentration decreased. This might be due to degradation of cellulase by proteolytic enzymes released by the fungus. The presence of mild proteolytic activity was confirmed in the cell free filtrate. The maximum CMase (CX) activity was shown by *M. verrucaria* (4.42 units/ml) upto 72 hr.

Corn cobs, a cellulolytic agricultural waste, can effectively be utillized as a carbon source for single cell protein production by cellulolytic fungi.

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On Wet Milling Suitability of Four Maize Cultivars

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Four Indian maize cultivars—'Amber Composite', 'Deccan 101', 'Ganga 5' and 'Vijay' were assessed for their wet milling suitability. The yield and the recovery of starch were satisfactory. Protein content in the extracted starch was below the tolerence limit. Lower contents of starch in the grain might be the cause for just satisfactory results in the wet milling. However, these varieties could be used as source materials in wet milling industry in India.

In India wetmilling industry was by far the major single consumer of maize. There are well known varieties developed in the country suitable for starch production, like 'Hi-Starch', 'Ganga Safed-2', etc. It has been observed that consumption of starch and starch-based products in India has increased in the last decade and the trend is towards further increase[1]. There were about ten major starch manufacturing companies in India, dealing with wetmilling of maize for the production of native starch and in some, further processing to its modified and/or derivative substances. There is little published literature on the wet milling of Indian maize varieties. Therefore, a preliminary study was initiated in our laboratory to determine suitability of four Indian maize cultivars for wet milling.

Materials and Methods

Four maize varieties—'Amber Composite', 'Deccan 101', 'Ganga 5' and 'Vijay', grown in Hyderabad, Andhra Pradesh, India, during *Rabi* 1977 were used in this study. This material was grown in single plots, and was supplied by the All India Coordinated Maize Improvement Project, New Delhi.

The procedure used in wet milling was essentially that of Pelshenke and Lindemann[2] and Kempf and Tegge[3], which was standardized in this laboratory showing good correlations between the yields in this method and the industry processing of maize for starch. A representative sample, obtained by quartering in Burrows sample-divider, of 50 g of maize kernels were steeped in water containing 0.2 per cent sulphur dioxide for 50 hr at 50°C. The steeped maize was carefully degermed by hand. Germs were dried, weighed, and the oil content as well as quality were determined. The degermed maize was ground in a laboratory grinder-mixer and hulls (size $>150\,\mu$) were separated. The resultant starch-protein suspension was centrifuged, washed and the concentrated slurry was separated into starch and gluten (protein) fractions on 'starch-tables' imitating those in the industry. Fractions of starch, gluten and hulls thus obtained were dried overnight and weighed.

Whole grain as well as the separated components were analyzed for chemical constituents using standard procedures[4]. Viability and bulk volume were estimated as per the standard procedures[5].

Results and Discussion

Physical properties and chemical constituents: Data for all the four cultivars are presented in Table 1. As revealed by the per cent viable kernels, the grains are sound and healthy. Variation in 1000-kernel weight and bulk volume was conspicuous compared to viability.

Starch content was nearly the same in all the cultivars, averaging to 69 per cent. 'Deccan 101' which had the highest starch content amongst the four cultivars. also had the highest protein content but low fat content. Some variation in protein and fat contents were noted. The ranges of crude fibre and mineral matter were 2.04-2.23 per cent and 1.53-1.72 per cent respectively, inferring not much variation amongst the cultivars. Similar trend was noted with respect to pH of the grain and acidity, except in 'Ganga 5', wherein the acidity was higher compared to the rest of materials.

Starch yield and recovery rate: Fig. 1 indicates the yield and recovery of starch in wet milling, as well as the respective classification pertaining to them[5].

The four maize cultivars gave an average of 63 per cent (range 62.3-63.6 per cent) starch yield and 91.4 per cent (range 90.1-92.3 per cent) starch recovery rate. As per the classification indicated in Fig. 1, these varieties

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TABLE 1. PHYSICAL PROPERTIES AND CHEMICAL ANALYSIS OF MAIZE VARIETIES

Variety	Viability (%)	1000-kernel wt. (g)	Bulk volume (ml/1000-kernels)	Crude starch (%)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Mineral matter (%)	Acidity (%)	pH
Amber Composite	99	237	294	69.1	12.4	4.68	2.14	1.66	1.45	6.31
Deccan 101	100	233	287	69.2	12.8	4.64	2.05	1.72	1.28	6.37
Ganga 5	100	291	364	69.0	11.6	4.83	2.23	1.53	2.45	6.14
Vijay	97	276	342	68.6	11.9	5.43	2.04	1.61	1.45	6.43

scored only 'satisfactory' in their wet milling suitability. The main reason can be attributed to the relatively lower contents of starch in the source material itself. As seen from Fig. 1, no appreciable difference could be found either in the starch yeield or recovery rate amongst the four maize cultivars.

Protein content in the extracted starch was below the maximum value of 0.6 per cent as prescribed in German Starch Standards[6]. 'Ganga 5' had the highest protein content (0.59 per cent), followed by 'Amber Composite', 'Vijay' and 'Deccan 101'.

Yields of by products: The data on the chemical analysis of the by products and their yield as gluten, germ and hull as well as total soluble solids (TSS) in the steepwater are presented in Table 2. Though not much variation was noted in the yield of gluten, the differences in the protein content of gluten fraction was interesting. 'Ganga 5' and 'Amber Composite' gave higher protein compared to 'Deccan 101' and 'Vijay'. However, the protein content of the gluten can easily be increased by additional centrifugation, washing and concentration.

Yield of germ is same in all the varieties and also very limited variation was observed in the fat content of the germ. However, the acid number and free fatty acid (FFA) content (expressed as oleic acid) were different. 'Ganga 5' which had the highest acidity and lowest pH value in whole maize sample, surprisingly recorded the least acid number amongst the varieties, which needs

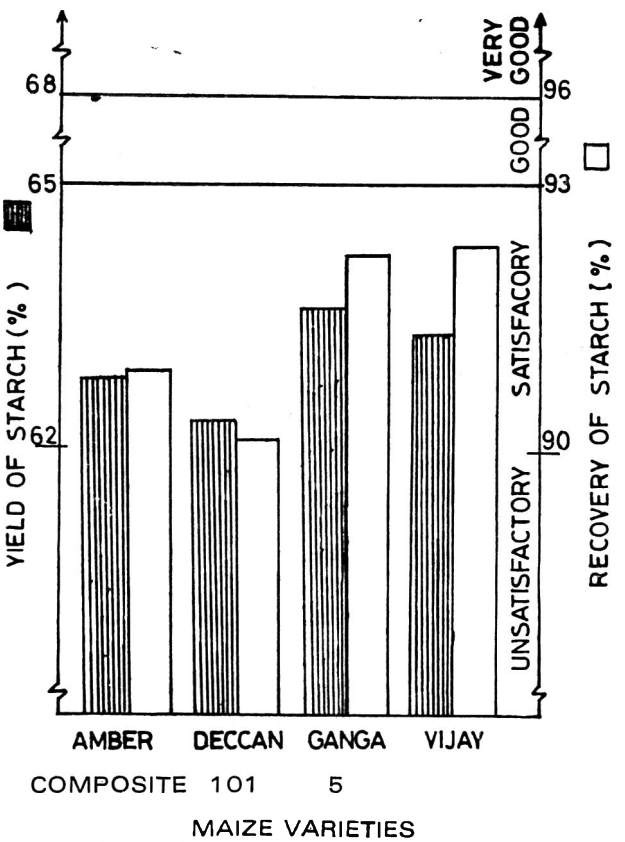

Fig. 1. Yield and recovery of starch from maize varieties (Limits specified for evaluation indicated)

TABLE 2. COMPONENT YIELDS IN WET MILLING IN RESPECT OF BY PRODUCTS (%, DRY BASIS)

Variety	Gluten		Germ				Hull		TSS in steepwater
	Yield	Protein	Yield	Fat	Acid No.	FFA	Yield	Starch	
Amber Composite	10.4	61.3	8.66	45.6	6.79	3.42	9.50	12.90	5.86
Deccan 101	10.8	55.7	8.60	46.8	7.07	3.56	8.80	8.84	5.93
Ganga 5	10.0	61.5	8.82	48.5	1.69	0.97	8.64	10.50	5.32
Vijay	10.7	54.3	9.14	50.6	5.58	2.80	8.38	10.50	5.62

further investigation. Acid number amongst the cultivars, was much variable compared to the FFA content. 'Vijay' recorded the second lowest in value in acid number, also ranked the same in FFA content, where as a change in situation was noted amongst 'Deccan 101' and 'Amber Composite'. With respect to total soluble solids percentage in the steep water, much variation was not observed amongst the cultivars.

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A Study of Interaction of Bread Improvers on Rheological and Baking Properties of Punjab Wheats

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Flours of five wheat varieties namely: 'WG 357', 'WG 377', 'PV 18', 'K 227' and 'S 308' were used for study of the interaction of slow and fast acting improvers on dough and baking characteristics. The dough characteristics were evaluated using Chopin Alveograph. The tightening effect of ascorbic acid and potassium bromate on doughs of various flours was well marked, as illustrated by decreased extensibility and increased stability. The strength of flour measured by the area under alveogram was invariably increased with the exception of S 308 where it was reverse. The optimised baking formula indicated the beneficial effect of potassium bromate but no such improvement was observed with the other two. There was no effect of any of the improvers on crumb characteristics using either of the test baking procedures. Generally potassium iodate was damaging to the baking quality of flours. The results of this study point to a complement of condition such as incorporation of fat and milk solids in the formula for the interaction of improver for obtaining higher loaf volume.

Increased production of wheat in the country necessitates the use of improvers in bread making. The baker has already realised the benefit derived from potassium bromate to improve the crumb grain and texture.

Only limited studies have been made so far on improving the baking quality of Indian wheats. Singh and Bailey[1] tried some studies on the quality of Indian wheats and their interaction with potassium bromate, using the 'Remix' baking procedure developed by Irvine and McMullan[2]. Some improvement in bread making qualities of Punjab wheats to potassium bromate has been observed by Finney *et al*[3]. Other chemicals like potassium iodate and ascorbic acid have not been so far investigated in our country.

Materials and Methods

Five commercially important varieties of wheat—'WG 357', 'WG 377', 'PV 18', 'K 227' and 'S 308' were obtained from the Punjab Agricultural University from

TABLE 1. PHYSICO-CHEMICAL CHARACTERISTICS OF FLOURS OF DIFFERENT VARIETIES OF WHEAT

Variety	Protein (%)	Ash (%)	Diastatic activity (mg maltose/10 g)	Proteolytic activity (HU/g)	Sedimentation value (cc)	Damaged starch (%)	Gluten (wet) (%)
WG 357	10.8	0.44	186	1.6	24.8	9.32	34.5
WG 377	9.6	0.48	278	1.5	25.6	13.2	35.3
PV 18	9.2	0.44	206	1.8	27.2	10.8	35.9
K 227	9.2	0.44	222	1.6	32.4	11.2	35.8
S 308	10.3	0.46	144	1.9	23.0	7.2	31.7

the 1973 crop. These were conditioned to 15 per cent moisture and milled on the Quadrumat Jr experimental mill after 48 hr of rest period. The composition of the flour is given in Table 1.

Fresh compressed baker's yeast (*Saccharomyces cerevisiae*) supplied by M/s Shaw Wallace and Co. Ltd., Bombay was used.

Potassium bromate, potassium iodate and ascorbic acid were of analytical grade and were used at 0, 10, 20 and 30 ppm levels on the flour basis and were added in the aqueous solution form.

All tests were carried out according to the standard AACC[4] procedures. Proteolytic activity was determined according to the Ayre-Anderson method as illustrated by Miller[5] and adopted by the AACC[4]. Alveograph curves were obtained according to the procedures illustrated by Kent-Jones and Amos[6]. The 50g bowl was used for the Chopin alveograph. AACC[4] straight dough test baking procedure and the optimized 'Rich' baking formula of Finney and Barmore[7] were used to study the effect of improvers in baking. In addition 0.5 per cent malt extract (60°L) as diastatic supplement was incorporated into the AACC formula. The doughs were optimally mixed at the standardized baking absorptions. Loaf volumes were measured by the rape seed displacement method and were assessed for quality parameters such as crumb texture, grain crust colour, etc.

Results and Discussion

Effect of improvers on the rheological properties of flours: The results of extensibility (length), resistance to extension (height) and baking strength (area) of flours treated with various improvers as studied with alveograph are given in Table 2. There was a marked vari-

TABLE 2. EFFECT OF BREAD IMPROVERS ON ALVEOGRAPH CURVE CHARACTERISTICS

Variety	Alveograph curve characteristics	Ascorbic acid (ppm)				Potassium bromate (ppm)				Potassium iodate (ppm)			
		0	10	20	30	0	10	20	30	0	10	20	30
WG 357	L (mm)	93	92	73	64	93	99	95	93	93	61	51	39
	P (mm)	55	56	52	55	55	52	51	19	55	50	44	42
	A (cm^2)	23.2	25.0	22.3	20.1	23.2	24.7	24.1	21.0	23.2	16.2	11.0	9.4
WG 377	L (mm)	52	65	53	50	52	68	73	66	52	49	30	28
	P (mm)	78	76	84	85	78	63	62	61	78	64	63	54
	A (cm^2)	25.2	29.2	27.4	26.6	25.2	20.7	24.2	19.5	25.2	18.3	12.6	10.2
PV 18	L (mm)	69	74	59	58	69	69	75	73	69	62	44	37
	P (mm)	64	60	55	67	54	57	56	59	54	58	56	55
	A (cm^2)	21.2	23.3	19.2	20.0	21.2	22.5	21.4	22.8	21.2	20.2	14.6	13.2
K 227	L (mm)	76	59	65	48	76	62	70	66	76	64	44	31
	P (mm)	60	70	70	74	60	70	63	62	60	64	63	55
	A (cm^2)	24.6	23.5	22.8	21.2	24.6	29.1	25.2	23.5	24.6	22.6	18.7	10.3
S 308	L (mm)	61	64	63	65	61	58	60	50	61	54	19	15
	P (mm)	55	56	58	56	55	55	56	54	55	51	50	45
	A (cm^2)	22.4	19.0	14.5	16.1	22.4	15.2	15.0	14.3	22.4	12.0	6.2	5.0

L—Extensibility P—Stability A—Baking strength

TABLE 3. EFFECT OF IMPROVERS ON THE LOAF VOLUME (CC) OF DIFFERENT VARIETIES OF WHEAT BY THE AACC—TEST BAKING PROCEDURE

Variety	Ascorbic acid (ppm) level				Potassium bromate (ppm) level				Potassium iodate- (ppm) level			
	0	10	20	30	0	10	20	30	0	20	30	30
WG 357	575	550	565	530	575	490	490	475	575	510	470	390
WG 377	475	480	460	480	475	450	420	420	475	440	350	325
PV 18	475	490	510	510	475	500	485	470	475	445	410	345
K 227	525	535	500	500	525	460	470	475	525	490	420	370
S 308	475	465	460	475	460	455	450	450	475	455	390	370

ation in the curve of different flours. Flours without any improver exhibited marked varietal differences in the curve parameters. The extensibility of doughs of different varieties was found to be 93 for 'WG 357', 52 mm for 'WG 377', 69 mm for 'PV 18', 76 mm for 'K 227', and 61 mm for 'S 308', with corresponding heights of 55, 78, 54, 60 and 55 mm, respectively. Extensibility decreased generally with 20 and 30 ppm of ascorbic acid in all varieties except 'S 308', where slight increase (4 mm) was observed. When different varieties were compared, marked decrease in extensibility occurred in variety 'WG 357' from 93 to 64 mm and of 'K 227' from 76 to 48 mm with increased dosages of 0-30 ppm acid. In the case of 'PV 18', the corresponding decrease was from 69 to 58 mm. The effect of potassium iodate on the alveograms was more pronounced. Extensibilities decreased markedly as the dosage was increased from 0 to 30 ppm. The extensibility of 'WG 357' decreased from 93 to 39 mm, of 'WG 337' from 52 to 28 mm, of 'PV 18'. from 69 to 37 mm, of 'K 227', from 76 to 31 mm, and of 'S 308' from 61 to 15 mm.

The curve area decreased considerably (22.4 to 14.3 cm^2) for the dough of 'S 308' treated with 30 ppm of potassium bromate and from 22.4 to 5.0 sq. cm at the same level of potassium iodate. Curve heights increased in case of 'PV 18' and 'WG 357' treated with 30 ppm of ascorbic acid or potassium bromate. The adverse effect of potassium iodate on the curve areas of each variety was in contrast to the effects of ascorbic acid and potassium bromate. The worst affected dough was that of 'S 308'. These observations point to the limitation of flours towards the fast acting oxidizing improvers which instead of effecting improvement, might prove deleterious to the baking quality of flours.

Effect of improvers on the baking quality of flours: The results of AACC test baking supplemented with 0-30 ppm of each of the improvers are given in Table 3. The results of optimized baking test with 0 and 20 ppm of each improver used are shown in Table 4. 'PV 18' flour seemed to have responded to ascorbic acid as indicated by increased loaf volume from 475 to 510 cc when dosages of 10 and 20 ppm of the improver were used in straight dough AACC baking procedure. Flours of varieties 'S 308', 'K 227', and 'WG 357' showed no response to ascorbic acid, whereas slight decrease in the loaf volume of 'WG 357' was recorded. The loaf volumes in all cases except 'PV 18' decreased when tested for their response to potassium bromate. The effect of potassium iodate on loaf volumes was even more detrimental precluding its use for improving the baking quality of flours. The results are in line with those obtained for doughs using alveograph.

The baking results of optimized formula brought out the response of flours of different varieties to potassium bromate. The loaf volume increased by 15 to 40 cc when 20 ppm of the improver was used. The maximum response has been shown by 'K 227', and the least by 'WG 377'. The loaf volumes obtained by using optimised

TABLE 4. EFFECT OF IMPROVERS ON THE LOAF VOLUME (CC) OF DIFFERENT VARIETIES OF WHEAT BY OPTIMIZED BAKING PROCEDURE

Variety	Ascorbic acid (ppm) level			Potassium bromate (ppm) level			Potassium iodate (ppm) level		
	Optimised baking		AACC method	Optimized baking		AACC method	Optimized baking		AACC method
	0	20	20	0	20	20	0	20	20
WG 357	535	550	575	585	560	575	535	540	575
WG 377	575	555	475	575	590	475	575	530	475
PV 18	560	580	475	560	590	475	560	560	475
K 227	560	555	525	560	600	525	560	560	525
S 308	535	540	475	535	565	475	535	530	475

baking formula, with and without the improver, far exceeded the loaf volume obtained by the lean AACC straight dough method designed to measure differences in protein quality as reflected by loaf volumes. The results are in close compromise to those of Hoseney *et al*[8]. who got no bromate response with the defatted flour.

The loaves generally had dark crusts, but those treated with potassium iodate, were torn and highly unattractive. The crusts of 'WG 377' were specially dark brown which might be attributed to high damaged starch content of the flour. The loaves of 'WG 357' were consistently rated superior to others as regards volume and crumb texture and grain. The crumb was rated softer than those of the other varieties. The loaves of 'PV 18' were not so soft. 'S 308' loaves were distinctively of whiter crumb in comparison to other varieties.

Both crust colour and shredding were dependent on variety and formula ingredient but shredding was also affected to some extent by the improvers as indicated by increased shredding in case of AACC formula baked loaves. But it was totally lacking in optimized formula loaves. No improvement was observed while using the improvers. Crust colour of loaves baked with optimized formula was comparatively darker. Ascorbic acid supplementation in AACC formula had no appreciable effect on crumb texture and grain, except for the variety 'S 308' where 30 ppm of the improver resulted in medium grain as compared with the fine grain of the control. Potassium bromate and potassium iodate produced an over all deterioration of the crumb texture and grain with increasing dosages of supplementation to varying extents, but more so with potassium iodate. The softer texture was transformed to a harder one, and finer crumb grain to a comparatively coarser one with the addition of these improvers. On the other hand there was no alteration in these two characteristics when these improvers were used as supplements in the optimized baking formula. A deterioration of crumb grain was due to the non uniform retention of gas owing to the stiffness of dough which failed to expand uniformly. The harder texture was the consequence of reduced loaf volume and coarser crumb grain. It is a well known fact that the doughs which fail to rise to a sufficient level during proving produce smaller and stubby loaves with hard textures, comparable with the present observations.

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Baking Response of Punjab Wheats to Proteolytic Supplementation

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Flours milled from five wheat varieties namely; 'WG 357', 'WG 377', 'PV 18', 'K 227' and 'S 308', were used for the study. The proteolytic activity (HU/g) of the flours was very low and ranged between 1.5 and 1.9. The effects of different levels of proteolytic supplements consisting of RhozymeA-4, papain and malt extract on the rheological properties of dough were studied using the mixograph and alveograph. Elevation in the mixing requirements of 'K 227' flour with increased amount of the proteases compared with decreased mixing times of weaker flours e.g. 'S 308' was noteworthy. Mixing tolerance was increased in all the flours but the strength of the dough as measured by the height of the mixogram was decreased. Alveograms indicated a decrease in the strength of dough with no effect on the extensibility. The baking strength was improved in some cases with lower dosages of the supplements. Loaf volumes of various flours tended to increase, specially with malt extract but the most significant improvement was in the crumb grain, slicing properties and symmetry of loaves, desirable in commercial baking. Higher dosages of papain were deleterious to the loaf volume as well as other characteristics and also the taste which turned bitter.

The activity of proteases in wheat though reported as quite low in comparison with animal proteolytic enzymes, nevertheless, is of considerable importance for normalgluten behaviour in a dough. The use of proteolytic enzymes in baking bread from strong flours assumed major significance as the bakers began to replace malt flour supplements with fungal amylases deficient in proteases[1]. It has been observed by Read and Haas[2] and Yoshinaka and Shizunori[3] that the physical properties of wheat gluten were modified by the action of proteolytic enzymes causing fluidity of gluten in the dough, but as reported by Pomeranz *et al*[4]. different enzymes differ markedly in their effect on the rheological and baking properties of flour. Bains and Irvine[5] reported that the indigenous wheats produce short and tight doughs as compared with the North American wheats and attributed this phenomenon to the deficient proteolytic acitivity and coagulation of gluten proteins due to the higher temperatures during harvest.

Materials and Methods

The flour samples were the same as used by the authors[6] in an earlier study. Fungal protease (Rhozyme A-4) was obtained from M/s Rohm & Haas Co., U.S.A. Papain from E. Merck & Co., Germany and malt extract from M/s Fleischmanns, U.S.A. Rhozyme A-4 and papain were added at levels of 0.0, 1.25, 2.50 and 5.00 mg and malt extract at levels of 0.0, 0.15, 0.30 and 0.45 g on 100 g flour basis, in aqueous solution form.

All analytical and dough testing techniques employed were essentially the same as used earlier. Mixograph was operated according to the procedure illustrated by Kent-Jones and Amos[7] and the baking absorptions were used for the purpose. AACC[8] straight dough test baking technique was adopted for evaluating the effect of proteolytic supplements in baking.

Results and Discussion

Effect on mixogram characteristics: Mixogram parameters of different flours supplemented with proteolytic enzymes are shown in Table 1. The baking-mixing time either increased or remained unchanged with the increasing concentrations of proteolytic supplements except for 'S 308' with the increasing levels of various proteolytic supplements, and for 'WG 357' and 'WG 377' when supplemented with malt extract. In these cases decreases in mixing time were observed. The increase in mixing time was very pronounced in the case of 'K 277' flour with Rhozyme A-4 as the proteolytic enzyme source. It was observed that in the case of comparatively weaker flours mixing requirement diminished but on the other hand mixing requirements increased for stronger flours like 'K 227' having highest sedimentation value and gluten content[6].

Mixing tolerance was enhanced to varying degrees with all the supplements, for the varieties studied. The effect was more elaborate in case of 'WG 377' with malt extract at 0.45 per cent level, where it increased from

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TABLE 1. EFFECT OF PROTEOLYTIC SUPPLEMENTS ON MIXOGRAPH CURVE CHARACTERISTICS OF FLOURS

Variety	Dosage*		Rhozyme A-4				Mixograph curve characteristics				Malt extract			
	Rhozyme A-4 & papain	Malt extract	Mixing time	Height	Mixing tolerance	Dough development area	Mixing time	Height	Mixing development	Dough tolerance area	Mixing time	Height	Mixing tolerance	Dough development area
	mg/100g	g/100g	min	cm	∠°	cm²	min	cm	∠°	cm²	min	cm	∠°	cm²
WG 357	0.00	0.00	3.4	6.2	130	28.7	3.4	6.2	130	28.7	3.4	6.2	130	28.7
	1.25	0.15	3.2	6.2	135	26.7	3.5	6.0	139	31.3	3.0	6.4	139	26.3
	2.50	0.30	3.5	6.0	136	27.0	3.3	6.1	136	25.0	3.1	6.2	137	26.1
	5.00	0.45	3.6	6.1	134	29.3	3.7	6.1	132	34.3	3.2	6.3	136	28.5
WG 377	0.00	0.00	2.9	6.2	129	27.0	2.9	6.2	129	27.0	2.9	6.2	129	27.0
	1.25	0.15	2.8	6.0	133	25.4	3.0	6.2	131	28.2	2.8	5.2	151	23.0
	2.50	0.30	2.9	6.0	128	25.0	3.2	6.2	121	30.6	2.4	5.5	149	21.0
	6.00	0.45	2.8	6.0	134	26.0	2.7	6.3	134	25.1	2.4	5.3	152	20.0
PV 18	0.00	0.00	2.5	5.6	140	22.3	2.5	5.6	140	22.3	2.5	5.6	140	22.3
	1.25	0.15	2.6	5.5	150	26.0	2.6	5.6	147	26.0	3.7	5.5	144	29.0
	2.50	0.30	2.5	5.6	140	24.3	2.5	5.5	147	23.5	3.3	5.4	153	28.3
	5.00	0.45	2.5	5.5	149	23.7	2.5	5.6	145	24.6	3.5	5.5	148	32.5
K 227	0.00	0.00	2.9	6.1	146	28.2	2.9	6.1	146	28.2	2.9	6.1	146	28.2
	1.25	0.15	3.6	5.6	141	29.4	3.6	5.6	150	31.1	3.3	5.2	151	25.4
	2.50	0.30	3.7	5.5	153	32.6	3.4	5.9	140	27.7	3.4	5.4	147	28.3
	5.00	0.45	4.0	5.5	150	33.4	3.3	5.7	144	27.2	3.6	5.5	143	29.3
S 308	0.00	0.00	1.7	6.6	115	18.0	1.7	6.6	115	18.0	1.7	6.6	115	18.0
	1.25	0.15	1.5	6.5	107	18.0	1.5	7.0	127	16.4	1.4	6.7	120	15.0
	2.50	0.30	1.4	6.5	120	17.0	1.4	6.9	127	16.7	1.4	6.5	127	16.4
	5.00	0.45	1.5	6.2	125	19.0	1.4	6.8	123	16.4	1.3	6.8	113	13.4

*For respective treatments.

130 to 152°. The increase in mixing tolerance through proteolytic supplementation is obvious as it results in better dough development which is able to resist breaking due to over work, to a greater extent.

The change in the height of mixograms was not appreciable except for 'K 227' in which it was reduced by proteolytic supplements. Both 'WG 377' and 'K 227' when treated with malt extract at 0.15 per cent level, the height was reduced to 5.2 cm from 6.2 and 6.1 cm respectively. In other cases either the height decreased a little or remained unchanged. The decrease in mixogram height may be attributed to the mellowing effect of the proteolytic enzymes on wheat gluten, thereby reducing the strength of the dough and making it more pliable and extensible.

The variation in dough development area as measured from mixograms was a function of the variety and supplement and no generalization could be made. Noteworthy results were obtained with 'PV 18' when supplemented with malt extract and 'WG 357' with papain. In these cases, the respective areas increased from 22.3 to 32.5 cm² and from 28.7 to 34.3 cm². The rate of dough development decreased in all cases except for 'S 308' when treated with papain and malt extract where there was a little increase. The decrease in rate of dough development was very much obvious as the baking mixing times had increased and the height of the mixograms reduced slightly.

The rate of dough weakening seemed to decrease with the increased amount of proteolytic supplement upto medium level, but generally increased with the highest dosage. This trend was liable to variation due to variety and nature of proteolytic enzyme employed. This behaviour was in accordance with the results obtained for increasing tolerance. Since, increase in mixing tolerance is associated with a corresponding decrease in dough weakening rate and a decrease in the weakening angle is a desirable characteristic in baking due to the reduced risk of overmixing.

Effect on Alveogram characteristics: The alveogram

TABLE 2. EFFECT OF PROTEOLYTIC SUPPLEMENTS ON ALVEOGRAPH CURVE CHARACTERISTICS

Variety	Dosage*		Alveograph curve characteristics								
	Rhozyme A-4 & papain	Malt extract	Rhozyme A-4			Papain			Malt extract		
	mg/100g	g/100g	L mm	P mm	A cm²	L mm	P mm	A cm²	L mm	P mm	A cm²
WG 357	0.00	0.00	75	67	26.0	75	67	26.0	75	67	26.0
	1.25	0.15	95	75	36.5	93	68	29.5	84	66	28.3
	2.50	0.30	68	78	26.4	80	66	25.4	68	66	28.3
	5.00	0.45	54	75	47.5	55	63	23.5	58	67	28.1
WG 377	0.00	0.00	88	93	41.7	88	93	41.7	88	93	41.7
	1.25	0.15	81	90	39.5	64	79	32.0	47	77	22.0
	2.50	0.30	67	78	37.1	60	79	29.3	60	72	25.3
	5.00	0.45	75	79	34.4	58	74	2.53	44	77	24.5
PV 18	0.00	0.00	74	72	28.6	74	72	28.6	74	72	28.6
	1.25	0.15	68	68	25.5	74	77	30.3	70	67	26.7
	2.50	0.30	76	65	26.6	81	69	28.0	77	62	27.3
	5.00	0.45	80	67	27.4	78	59	22.5	57	62	22.6
K 227	0.00	0.00	80	73	29.2	80	73	29.2	80	73	29.2
	1.25	0.15	78	91	40.1	52	82	29.8	55	82	29.2
	2.50	0.30	86	76	34.0	68	77	32.1	53	73	24.0
	5.00	0.45	56	76	26.3	68	66	24.3	52	70	23.1
S 308	0.00	0.00	94	68	25.4	94	68	25.4	94	68	25.4
	1.25	0.15	83	66	25.0	62	67	24.3	86	65	24.0
	2.50	0.30	64	67	21.6	76	66	23.1	94	59	23.4
	5.00	0.45	78	63	20.6	61	62	18.0	93	53	21.2

*For respective treatments L—Extensibility P—Stability A—Baking strength

parameters as influenced by variety and proteolytic supplementation are given in Table 2. Extensibility of dough as measured by the length of the alveograph, generally, varied a little with the addition of different enzyme supplements to various flours. Only 'WG 357' with the lowest dosage of each supplement and 'PV 18' with the medium dosage of the supplements showed an increase in the dough extensibility over the respective controls. The change in extensibility of the dough is brought about by the softening effect of proteolytic enzymes on wheat gluten but excessive breakdown of gluten proteins make the dough short as has been observed by Johnson and Miller[9] and Kruger[10].

The strength of dough as measured by the height of alveogram was reduced with the increasing concentration of supplements. In case of 'K 227' and 'WG 357' a little increase was observed in the height of the curves, which vanished with the higher dosage. The decrease in dough strength was obvious as the proteolytic enzymes are known for their mellowing effect on bucky doughs, e.g. scission of peptide bonds thereby making the gluten weaker with shorter strands of proteins.

The baking strength expressed as area under the alveograms increased in case of 'WG 357' with the lowest dosage of the proteolytic supplements and in case of 'K 227' with lowest dosage of Rhozyme A-4 and papain but declined with the further increase in the amount of supplements. Malt extract had no effect on 'K 227' but in the remaining cases, the baking strength decreased gradually. The increase in baking strength was through increased extensibility of dough brought about with limited amount of proteolysis but on the other hand higher amount of proteolysis diminished it.

Effect on baking quality: Data in Table 3 indicate a beneficial effect of the supplements on loaf volumes, though not well marked in all cases. 'WG 357' flour when supplemented with 2.5 mg per cent of Rhozyme A-4 gave a loaf volume of 580 ml against 555 ml of the control. 'WG 377', 'PV 18' and 'S 308' wheats also gave loaves with increased volumes with the increased dosages of the enzyme. Variety 'K 227', however, showed no response to Rhozyme A-4 supplementation. Using the AACC straight dough method with 0.5 per cent added malt extract, the effect of Rhozyme A-4 was somewhat

TABLE 3. EFFECT OF PROTEOLYTIC SUPPLEMENTS ON LOAF VOLUME OF DIFFERENT FLOURS

Variety	Dosage*		Loaf volume (cc)			
	Rhozyme A-4 & papain mg/100g	Malt extract g/100g	Rhozyme A-4	Rhozyme A-4 with 0.5% malt extract	Papain	Malt extract
WG 357	0.00	0.00	555	545	568	555
	1.25	0.15	560	563	565	545
	2.50	0.30	580	545	585	565
	5.00	0.45	565	540	580	570
WG 377	0.00	0.00	435	463	485	435
	1.25	0.15	455	470	505	470
	2.50	0.30	465	478	480	475
	5.00	0.45	495	490	468	510
PV 18	0.00	0.00	420	473	483	420
	1.25	0.15	455	470	480	450
	2.50	0.30	460	473	493	470
	5.00	0.45	475	480	440	475
K 227	0.00	0.00	515	498	525	515
	1.25	0.15	515	515	510	520
	2.50	0.30	515	515	495	520
	5.00	0.45	520	518	440	520
S 308	0.00	0.00	410	445	465	410
	1.25	0.15	430	463	465	450
	2.50	0.30	430	458	470	440
	5.00	0.45	450	473	465	445
		C.D. (5%)	15.9	11.1	25.7	19.4

*For respective treatment.

obscured. There appears to be interaction of the variety to malt extract and Rhozyme A-4 supplements. The volumes of control loaves of different varieties increased due to malt extract supplementation, as by Rhozyme A-4 treatment, of some of the varieties, supporting involvement of proteolytic enzymes of malt, There was a deleterious effect of papain on the loaf volume of 'K 227' in contrast to the loaf volumes of the remaining varieties which showed no such definite trend. Malt extract supplementation had a fairly good effect on loaf volumes. The increase in loaf volume was the result of better gas retention due to the formation of elastic dough as revealed by the rheological measurements with Chopin alveograph. The more pronounced effect with the addition of malt extract may be ascribed to increased gas production due to the diastatic nature of the supplement besides being a source of proteolytic activity. Statistically the differences in loaf volume on account of different dosages of proteolytic supplements, were found to be significant and beneficial in all cases except that of papain, whose effect was deleterious.

The crumb grain and texture were definitely improved to varying degree in loaves of various test flours on proteolytic enzyme supplementation, with the exception of papain where both crumb texture and grain recorded deterioration. Presumably, improvement in grain is brought about through uniform retention of gas as a result of increased elasticity of dough as evidenced by the alveograms, whereas improvement in texture was the result of uniform and fine grain together with increased volume. There was no tangible change in crumb colour as a result of proteolytic supplementation.

There was considerable improvement in the slicing quality of loaves treated with proteolytic enzymes except for higher dosages of papain. The improvement in slicing was the result of improved grain structure and comparatively softer texture. As excessive dosages of proteolytic enzymes damage the grain structure and harden the texture, so there was deterioration in slicing quality of loaves with higher levels of papain. The improvement in slicing quality is a highly desirable feature in bread production. The results of this investigation point to the advantage of using the proteolytic supplement for this purpose.

The loaves produced with proteolytic enzymes showed smooth external finish and shredding was more or less

variety dependent. The loaves were more attractive in appearance because of symmetry, possible only if the dough is significantly mellowed. The 'S 308' loaves showed an edged appearance due to the flowy nature of the dough as its gluten is not strong. The colour of the exterior did not change with other enzyme sources except malt extract, which slightly darkened the exterior due to enhanced Mailard's reaction.

Finney and Fryer[11] in their studies on the hard red winter wheats observed that loaf volumes and mixing times decreased in general with temperatures above 90°F during the last 15 days of fruiting. Usually, harvest of wheat in Northern India falls at a time when high ambient temperatures prevail which damage gluten by incipient coagulation of proteins.

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Studies on the Use of "Ferripolyphosphate" for Isolation of Proteins from Different Whey Systems

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Proteins from the casein, rennet and *Paneer* whey systems of both buffalo and cow milks could be effectively precipitatedby tbe application of "Ferripolyphosphate." Whey proteins of buffalo milk were found to be more susceptible to precipitation under the influence of "Ferripolyphosphate" compared to those of cow milk. The final product obtained by this process was observed to be white, fluffy powder containing about 51.25 to 53.18 percent proteins and 11.16 to 15.11 percent iron. While the products obtained from the casein and rennet whey systems were electrophoretically similar, they differed considerably from those obtained from the *Paneer* whey systems. Chromatography on Sephadex G-75 showed that the process employed alters the elution profile of whey protein components.

Preparation of Ferripolyphosphate (FPP), a liquid complex of ferric ions with a long chain polyphosphate having Fe:P ratio of 1:12 has been described by Hazel *et al*[1]. This preparation has been found to be very effective for the precipitation of proteins from commercial acid whey by the cold precipitation technique[2]. The product so obtained has been shown to contain about 50 percent protein, 10 percent iron and 13 percent phosphorus in a highly assimilable form[3]. On the basis of these reports, it was felt that FPP could also function as protein precipitant for the whey systems available to the dairy industry in India. This investigation was, therefore, undertaken to study the conditions required for isolation of proteins from casein, rennet and *Paneer* whey systems of buffalo and cow milks and to evaluate certain aspects of the quality of product so obtained.

Materials and Methods

Samples of milk and whey were obtained as described in an earlier communication[4]. Moisture and fat were estimated according to the Majonnier modification[5]. Phosphorus was estimated according to the method of Meun and Smith[6]. Iron was estimated colorimetrically by the AOAC method[7]. Kjeldahl nitrogen was estimated as suggested by McKenzie[8]. Electrophoresis was carried out by the DPGE technique of Mathur and Srinivasan[4]. Chromatography on Sephadex G-75 was performed according to the method suggested by McKenzie[8], using a column of size 84 cm $\times$ 2.6 cm. FPP was prepared by the method of Jones *et al*[2].

Isolation of whey proteins: Samples of whey were arranged in ten lots of 100 ml each. Sufficient amount of FPP was added so as to provide ferric ion concentration of 0.01 to 0.10 M at regular intervals of 0.01 M.

The pH of the mixtures was then adjusted to 3.5 with (1:4) hydrochloric acid. Precipitated proteins were then centrifuged out at 5,000 g for 15 min. Proteins remaining in the supernatant were estimated by Kjeldahl nitrogen and protein components examined electrophoretically by the DPGE technique. Precipitated proteins were suspended in 10 ml of distilled water and dialyzed against distilled water for 24 hr at 4°C, with six changes of water. Dialyzed proteins were freeze dried and preserved for further analysis.

Results and Discussion

A screening test was carried out for determining the requirement of FPP for the precipitation of proteins from the casein, rennet and *Paneer* whey systems of buffalo and cow milks. The amount of proteins precipitated at different levels of added FPP from different whey systems of buffalo milk is illustrated in Fig. 1.

Protein precipitation from casein whey: With the increase in amount of added FPP, there was a progressive increase in the level of precipitated proteins from the casein whey of both buffalo and cow milks. It may be inferred from Fig. 1 that for the precipitation of 95 percent of proteins, requirement of FPP was 0.048 and 0.056 M as Fe for casein whey systems of buffalo and cow milks, respectively.

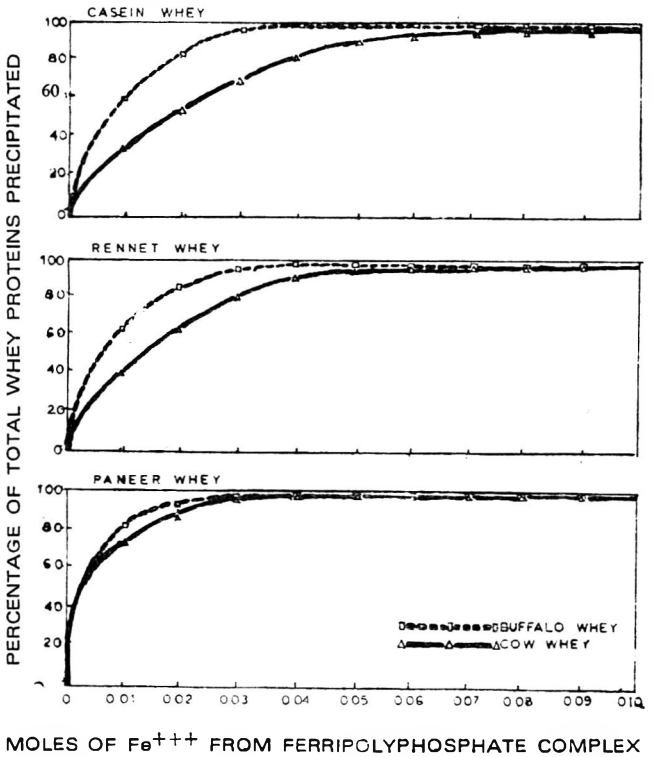

Fig. 1. Graph illustration of the effect of different levels of ferripolyphosphate complex on the precipitation of whey proteins from buffalo and cow milk whey systems

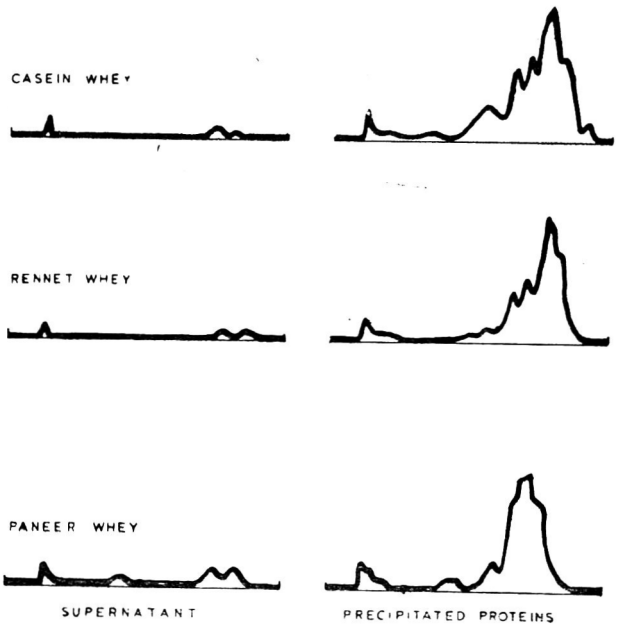

Fig. 2. Partition of buffalo whey protein components in the supernatant and precipitated proteins under the influence of ferripolyphosphate complex

Electrophoretic studies of the protein components present in the supernatant and precipitated proteins indicated that almost all components are precipitated effectively under the influence of FPP (Fig. 2 and 3).

Protein precipitation from Rennet Whey: Similar to the casein whey, there was observed a progressive increase in the amount of proteins precipitated as the level of added FPP was increased up to 0.03 M iron. Further addition of FPP did not bring about proportional increase in the amount of proteins precipitated. It may be

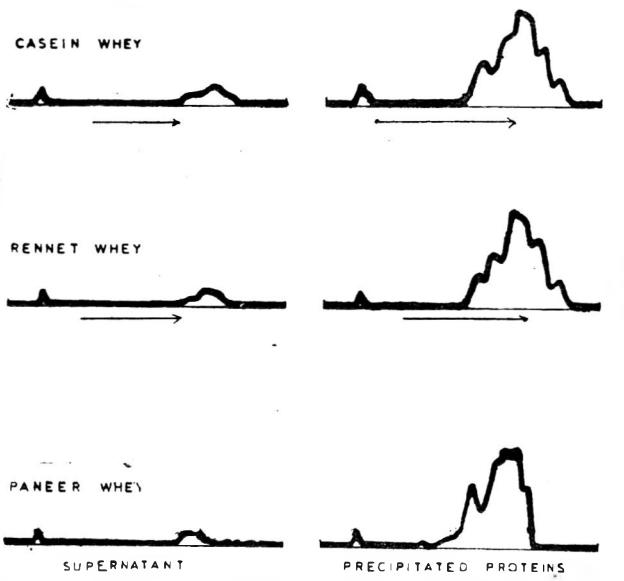

Fig. 3. Partition of cow whey protein components in the supernatant and proteins under the influence of ferripolyphosphate complex

TABLE 1. GROSS COMPOSITION OF WPI PREPARED FROM DIFFERENT WHEY SYSTEMS OF BUFFALO MILK BY THE APPLICATION OF "FPP" FOR COLD PRECIPITATION

Constituent	Casein whey	Rennet whey	*Paneer* whey
	(%)		
Proteins	51.25±0.44	52.18±0.73	52.67±0.88
Fat	nil	0.03±0.01	0.03±0.00
Ash	44.36±0.76	42.95±0.24	41.12±0.46
Moisture	3.20±0.00	3.17±0.00	3.46±0.00
Iron	15.11±0.68	13.29±0.54	11.16±0.62
Phosphorus	24.31±0.38	22.11±0.68	21.19±0.51

inferred from Fig. 1 that for the precipitation of 95 per cent proteins from the rennet whey systems of buffalo and cow milks, the requirement of FPP was 0.036 and 0.056 M iron respectively.

DPGE studies of the supernatant and precipitated proteins indicated that all components of proteins present in the rennet whey systems of both buffalo and cow milks were equally susceptible to precipitation under the influence of FPP (Fig. 2 and 3).

Protein precipitation from Paneer Whey: As may be observed from Fig. 1, proteins present in the *Paneer* whey system of both buffalo and cow milks were precipitated to a greater extent compared to those of casein and rennet whey systems, specially at the lower levels of added FPP. For precipitation of 95 percent proteins from *Paneer* whey system, the requirement of FPP was found to be 0.030 and 0.035 M as iron for the buffalo and cow milks, respectively.

Electrophoretic studies of the supernatant and precipitated proteins indicated that all the protein components present in the *Paneer* whey systems of both buffalo and cow milks could be effectively precipitated under the influence of added FPP.

From the data obtained during the course of this investigation, it was observed that irrespective of the whey system, whey protein components of buffalo milk were slightly more susceptible to precipitation under the influence of FPP compared to those of cow milk. In this manner, the requirement of FPP for the precipitation of equivalent amounts of proteins from whey systems of buffalo milk was slightly less compared to those of cow milk.

TABLE 2. GROSS COMPOSITION OF WHEY PROTEIN ISOLATES PREPARED FROM DIFFERENT WHEY SYSTEMS OF COW MILK BY THE APPLICATION OF "FPP" FOR COLD PRECIPITATION

Constituent	Casein whey	Rennet whey	*Paneer* whey
	(%)		
Proteins	52.68±0.52	52.79±0.38	53.19±0.42
Fat	nil	0.01±0.00	0.02±0.00
Ash	41.79±0.66	41.39±0.62	42.41±0.64
Moisture	3.10±0.01	3.22±0.01	3.15±0.00
Iron	12.82±0.46	13.10±0.87	12.71±0.31
Phosphorus	22.12±0.33	22.09±0.62	21.29±0.71

Recovery of whey proteins by other processes employing sodium hexametaphosphate[4], CMC[9] and alcohols[10] has been reported to range between about 55 and 80 percent. Based on these reports, application of FPP appears to be a comparatively superior process. Comparable levels of protein recovery have been attained by Block and Bolling[11] by the application of ferric chloride. However, the product obtained by the application of ferric chloride has been reported to be creamish to dark tan in colour.

Composition of "Whey Protein Isolates" (WPI) obtained from different whey systems: Gross composition of WPI obtained from different whey systems is given in Tables 1 and 2. It may be observed that WPI obtained by using FPP were particularly high in the ash content. In view of the high iron content, such preparations may be considered to be of considerable interest for the fortification of foods with protein and iron. In bioassay, such WPI has been found to be 92 to 100 percent efficient relative to ferrous sulphate, in restoring hemoglobin levels of iron-depleted rats and chicks[3]. However, ash content of WPI prepared by hexametaphosphate comp-

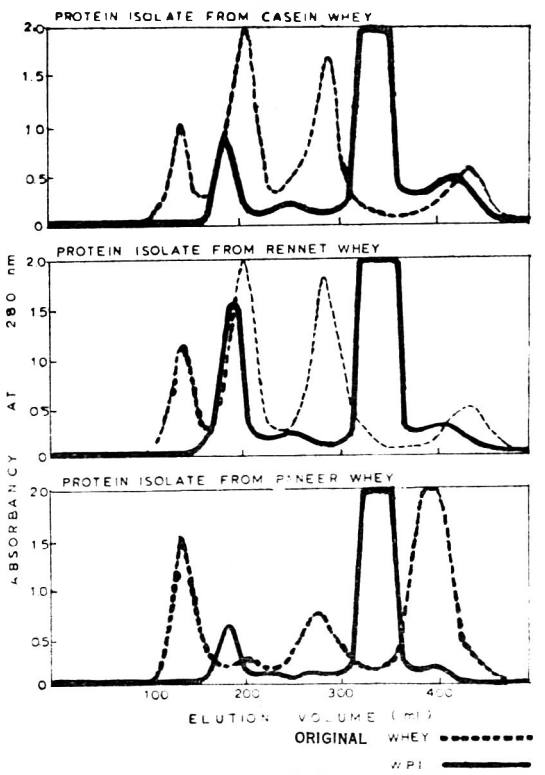

Fig. 4. Elution profile of whey protein isolates obtained by cold precipitation with Ferripolyphosphate complex from buffalo milk whey systems on Sephadex G-75 column (2.6×84 cm) at pH 6.3.

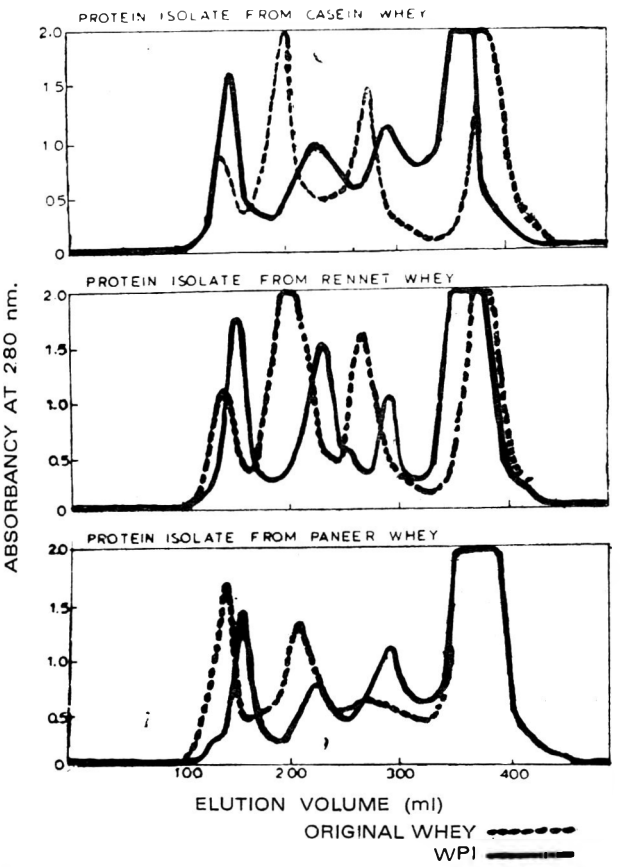

Fig. 5. Elution profile of whey protein isolates obtained by cold precipitation with ferripolyphosphate complex from cow milk whey systems on Sephadex G-75 at pH 6.3.

lexing, ferric ion complexing, CMC complexing, alcohol precipitation, Gel filtration and Ultra filtration processes has been reported to be comparatively quite low in the ash content[10-12].

Electrophoretic behaviour of WPI: The effect of the cold precipitation process by using FPP on the native properties of whey proteins was evaluated by studying the electrophoretic behaviour of WPI (Fig. 2 and 3). It was observed that the components of whey proteins present in the whey systems and the WPI displayed similar electrophoretic mobilities. It appears that the process of isolation employed in this study does not affect the electrophoretic behaviour of whey protein components. Further, it was observed that the WPI obtained from the casein and rennet whey systems were quite comparable electrophoretically but differed considerably from the WPI obtained from *Paneer* whey systems. These differences are attributable to the different state of whey proteins present in *Paneer* whey as a result of heat treatment (90°C) given to milk for manufacture of *Paneer*.

Chromatographic behaviour of WPI on Sephadex G-75: The elution profile of WPI obtained from various whey systems using FPP (Fig. 4 and 5) was found to be quite dissimilar from the whey protein profile of different whey systems. The distribution of proteins under different peaks was altered considerably both in the case of buffalo and cow milks. These observations indicate that when whey proteins are precipitated under the influence of FPP, different components of whey protein undergo certain molecular rearrangements. These changes became apparent when WPI were eluted at pH 6.3 in imidazole-HCl buffer (I=0.043).

However, different pH conditions, ionic strengths and resolving forces applied during the DPGE (stacking gel 5 per cent poly acrylamide, pH 6.0, I=0.013 and running gel 7.5 per cent poly acrylamide, pH 8.9, I=0.44) seem to overcome the protein—ion-protein interactions observed during chromatography on Sephadex G-75 and appear as separate entities. In this manner, differences in the Sephadex chromatoprahic and electrophoretic behaviours may be attributed to different state of equilibrium of WPI components with respect to the pH, ionic strength and resolving power of the two analytical systems.

WPI obtained by employing FPP different whey systems of both buffalo and cow milks were observed to be white fluffy powders with moderately good solubility in water. Based upon the quality of product obtained and good recovery of proteins from different whey systems, this process seems to have potential for pilot scale operations.

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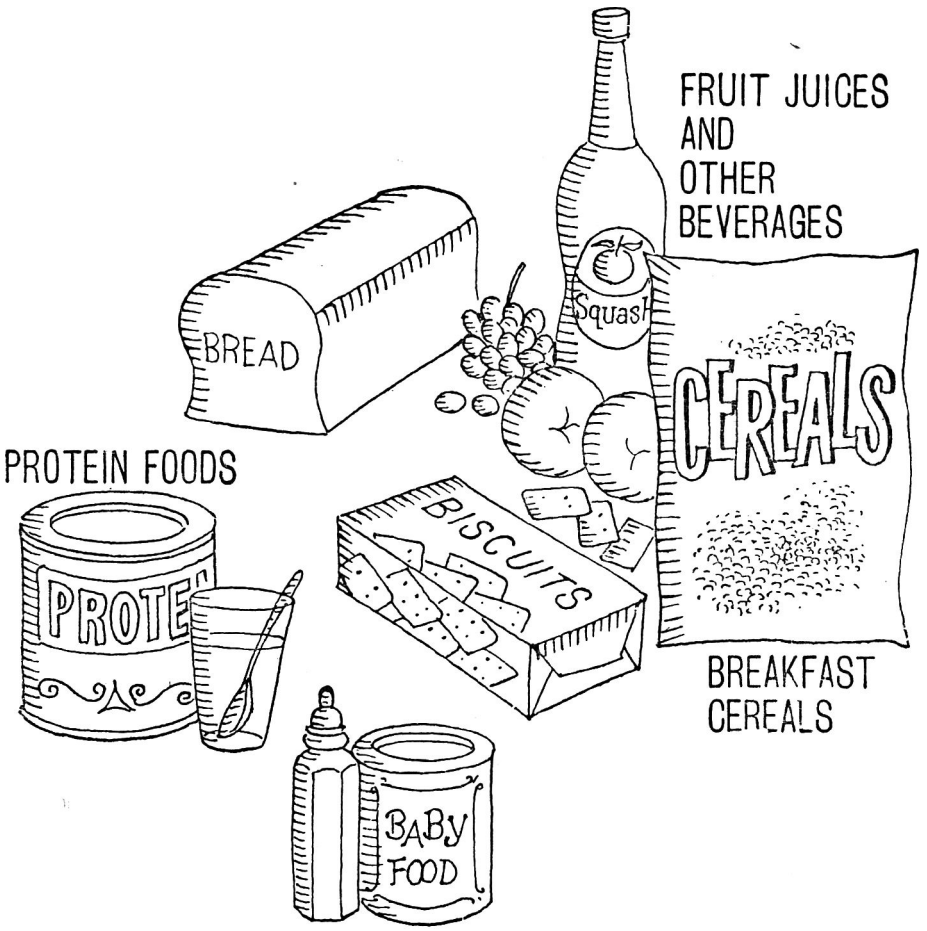

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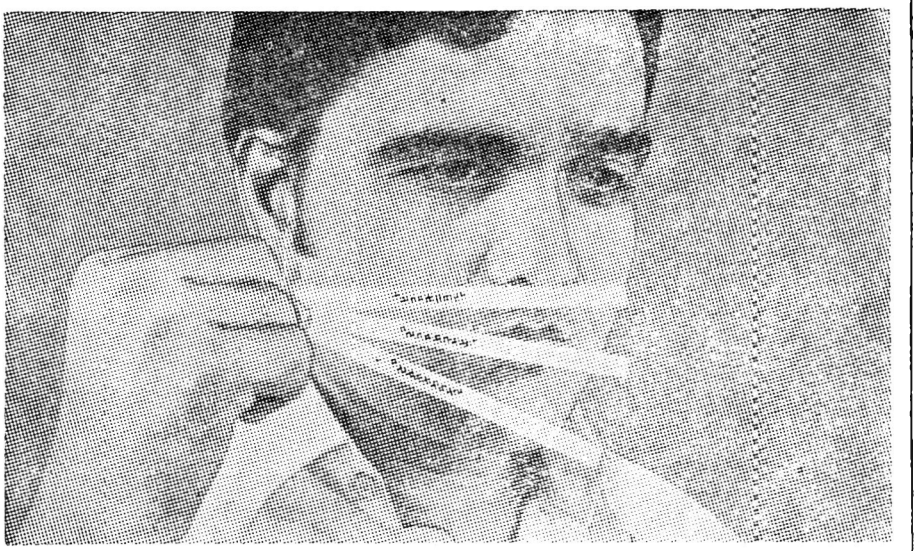

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