ENZYME REACTIONS AS INDEX OF FRESHNESS OF FISH AND SHELLFISH

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CERTIFICATE

This is to certify that this thesis entitled 'Enzyme reactions as index of freshness of Fish and Shellfish' is an authentic record of the work carried out by Sri. D. Damodaran Nambudiri under our supervision in the Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, in partial fulfilment of the requirements for the award of the Ph.D degree of the Cochin University of Science and Technology and that no part thereof has been presented before for any other degree in any University.

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ABBREVIATIONS

ADP	-	Adenosine - 5' - diphosphate
AMP	-	Adenosine - 5' - monophosphate
ATP	-	Adenosine - 5' - triphosphate
FFA	-	Free fatty acid
IMP	-	Inosine - 5' - monophosphate
LDH	-	Lactate dehydrogenase
NAD	-	Nicotineamide adenine dinucleotide
PV	-	Peroxide value
TBA	-	Thiobarbituric acid
TBC	-	Total bacterial count
TCA	-	Trichloro acetic acid
TMA	-	Trimethyl amine
TVN	-	Total volatile nitrogen

CONTENTS

PREFACE

For standardised quality control of fishery products, an objective criterion is necessary. A prerequisite in objective assessment of fish quality is adopting a clear distinction between freshness tests and spoilage tests. In the assessment of fish quality, freshness should be limited to the interval from the point of animal death to the first detectable signs of spoilage, after which the term freshness no longer applies. An objective test capable of measuring that critical range in terms of storage time and temperature, would be a valuable tool in quality control. It need not relate directly to flavour or texture of fish although these are major factors deciding consumer acceptability of fishes. Of the many methods available, freshness of fish can best be measured in terms of declining functional integrity in some components of the tissue. The reduction in enzyme activities in frozen stored fish flesh and its correlation with loss of freshness have an area of extensive investigation without much positive results.

An attempt has been made in this study to screen some fish muscle enzymes to assess their potential worth in testing the degree of freshness of fish. A problem with routine enzyme activity determinations is the complexity of the method of enzyme assay. Hence, in the present study as far as possible simple assay techniques were adopted. Several species were screened to assess the possibility of employing this procedure on a large scale. It is hoped that findings of this study will lead to the development of meaningful criteria in testing the freshness of fish.

This thesis has been divided into five chapters. The thesis starts with a review of work reported on the enzymes selected for the study. Object-

ive and plan of action of this study are described towards the end of this chapter. A precise account of the materials used and methodology adopted in the present study has been given in Chapter 2. It is followed by chapter 3 describing the effect of ice storage on activity of selected enzymes in fish and shellfish. Chapter 4 gives results of a study on stability of enzymes in fish and shellfish subjected to frozen storage. While carrying out the above work it has been observed that fishes undergo rapid stiffening while subjected to chilling. A detailed study has been carried out on this phenomenon and the results are presented in chapter 5 entitled "cold shock reactions in tropical fishes." A brief summary of this study and conclusions arrived at are presented at the end.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Post mortem degradation of fish muscle proceeds through rigor-mortis, resolution of rigor-mortis, autolysis and various other chemical/microbial changes. Most of the past studies on freshness of fish were based on the view that freshness of fish is mainly decreased by bacterial action. Shigeo Ehira (1976) experimentally elucidated the differences between bacterial freshness and freshness lowering due to bio-chemical changes. He has shown by experiments carried out on plaice and skipjack on ice storage that even while bacterial count, the amounts of total volatile nitrogen, trimethyl amine nitrogen and pH did not register any marked change, adenosine triphosphate and its degradation products exhibited drastic changes before putrefaction of fish. Similar experiments were performed on aseptic fish muscle which was iced and its freshness lowering examined as compared to non aseptic sample. Changes in the biochemical indices of freshness of fish were almost same for aseptic and nonaseptic samples. The author has confirmed that freshness lowering of fish was caused by rather auto-degradation of its tissues than bacterial action. Freshness of fish therefore must be considered to be closely related with biochemical changes in fish before putrefaction.

Sikorski (1980) has described the main processes occuring in the proteins of fish after catching under the influence of endogenous and bacterial enzymes as well as changes in the characteristics of fish. These changes are given schematically below. It is shown that as a result of the hydrolytic reactions catalysed by enzymes from tissues themselves, the penetration of micro-organisms is facilitated and nutrients are formed which would promote bacterial growth.

MUSCLE OF FRESHLY CAUGHT FISH

Relaxed extensible sarcomeres, actin and myosin uncoupled, pH about 7

ATP $\longrightarrow$ ADP release of Ca^{2+} $\downarrow$ from sarcoplasmic reticulum

FISH MEAT IN RIGOR MORTIS

Partly contracted sarcomeres, actomyosin, pH about 6

Cathepsins, Ca^{2+} activated $\longrightarrow$ $\downarrow$ $\longleftarrow$ glucuronidase and other lysosomal

proteinase, collagenase enzymes

TENDER FISH MEAT

Partly hydrolysed sarcoplasmic proteins, slightly disintegrated sarcomeres, disrupted collagen structure, pH about 7

endogenous enzymes $\longrightarrow$ $\downarrow$ $\longleftarrow$ bacterial enzymes

AUTOLYSED FISH MEAT

Partly hydrolysed proteins, non-protein nitrogenous compounds, pH about 7

Many enzymes of carbohydrate metabolism have been employed in the past for investigations related to the post mortem deterioration of fish quality. Much work has been carried out in the past employing succinic dehydrogenase assay in testing the freshness of fish. Other enzymes of carbohydrate metabolism such as malic dehydrogenase, α-glycerophosphate dehydrogenase, glutamate oxaloacetate transaminase, etc. were also employed in the past as indices of freshness of fish. Activity of proteolytic enzymes such as cathepsin D, alkaline protease etc. was determined in fish in order to assess freshness during storage.

1.1 ICE STORAGE STUDIES

Less work has been reported on employing enzyme activity as a test for measuring freshness of ice-stored fish. Some work were done in the past employing enzymes associated with the break down of nucleotides. Post mortem biochemi-

cal changes of nucleotides are important autolytic reactions taking place in fish. In fish muscle adenine nucleotides (5-8 μ mole/g) occupy more than 90% of total nucleotides (Ikeda, 1980).

In fish muscle rigor-mortis is induced with a decrease in the ATP level of muscle (Partman 1965). The intensity of rigor-mortis is dependent upon the amounts of ATP decomposed per unit time (Yamanaka et al, 1978). Heber et al (1973) reported that the cause for the rapid disappearance of ATP in the fish muscle stored below freezing point might be the activation of adenosine triphosphatase by Ca^{2+} released as a result of cellular destruction caused by ice crystal formation.

Actin is a water soluble protein. One molecule of ATP is bound to it. Myosin exhibits ATPase activity. The ATPase activity of both actin and actomyosin is stimulated by Ca^{2+}. Mg^{2+} inhibits myosin-ATPase activity but stimulates actomyosin activity. ATPase (EC. 3.6.1.8, ATP pyrophosphohydrolase) catalyses the following reaction,

$$ATP + H_2O \rightleftharpoons AMP + Pyrophosphate$$

Ca^{2+} ATPase activity of myofibrillar proteins of the two species of fish such as carp and sardine gradually decreased during ice storage (Seki & Narita, 1980). Seki et al (1980) observed that the myofibrillar EDTA - ATPase of fish decreases by 70% after one day storage in ice. Ca^{2+} ATPase activity decreased to 50% of their initial level after 6 days storage in ice. However, in the case of stoneflounder and plaice, total actomyosin Ca^{2+} ATPase activity changed little during 14 days ice storage (Ehira & Uchiyama, 1979). Seki et al (1979) reported that Ca^{2+} ATPase activity of carp myofibril stored in an alkaline medium increased during storage.

The effect of storage of carp post mortem muscle at room temperature ($25^{o}C$) for 4 days on myofibrillar ATPase activity was determined by Seki & Watanabe (1982). It was found that Mg^{2+} ATPase activity first increased rapidly, then decreased. Little change was observed in Ca^{2+} ATPase activity.

Lowering of freshness and thermal stability of actomyosin ATPase activity in the dorsal muscle of various fish from Ryuku fishing ground at different temperatures were studied by Nishimoto & Miki (1979). Freshness was measured by estimating K values of the samples at 0^{o}, 10^{o}, 20^{o} & $30^{o}C$ whereas thermostabilities of actomyosin was measured at $25^{o}C$, 30^{o} and $35^{o}C$ by way of liberated inorganic phosphate. Freshness estimation index, K value was calculated from the following formula:

$$K\ (\%) = \frac{HxR + Hx}{ATP+ADP+AMP+IMP+HxR+Hx} \times 100$$

where HxR represents inosine and Hx stands for hypoxanthine. Values in μmoles/g will be used for this calculation. It is not necessary to determine separately ATP degraded compounds for the purpose of calculating K value, but determining the total amounts of ATP related compounds and that of inosine and hypoxanthine is sufficient for this purpose.

ATP degraded compounds are formed in fish muscle by the degradation of ATP through the auto catabolic path way,

$$ATP \longrightarrow ADP \longrightarrow AMP \longrightarrow IMP \longrightarrow HxR \longrightarrow Hx$$

Rate constants of freshness lowering and inactivation of actomyosin-Ca^{2+} ATPase activities were evaluated in the above study. Regardless of the species the freshness lowering rate increased with temperature. The thermo stabilities of actomyosin-Ca^{2+} ATPase activities of the samples were found

to vary with species.

Lipoamide reductase (EC.1.6.4.3 NADH : lipoamide oxido reductase) is a flavo protein component of the multi enzyme pyruvate dehydrogenase complex and 2-oxo glutarate dehydrogenase complex. The mammalian enzymes are of mitochondrial origin. This enzyme catalyses the following reaction

$$NADH + \text{lipoamide} \rightleftharpoons NAD^{+} + \text{dihydrolipoamide}$$

Lipoamide oxidoreductase functions physiologically in the re-oxidation of dihydrolipoic acid, bound in amide linkage to the amino group of a lysine residue in the transacetylase or trans succinylase, the electron acceptor is NAD^{+}. The lipoic acid is reduced as a consequence of the thiamine pyrophosphate dependent oxidative decarboxylation of α keto acids, pyruvate and α keto glutarate yielding acetyl - CoA and succinyl CoA respectively.

Substrate specificity of lipoamide reductase is very broad. A large number of catalytic reactions are possible between pairs of hydrogen donors and acceptors. Suitable hydrogen donors are dihydrolipoyl derivative, NADH and oxidised pyridine nucleotide analogs and suitable acceptors are oxidised lipoyl derivative, NAD and oxidised pyridine nucleotide analogs, K_3 Fe $(CN)_6$, 2,6, dichlorophenol indophenol and to an extent O_2. NADH - 2,6 dichlorophenol indophenol reductase activity is known as diaphorase activity and this reaction was employed throughout the present study. Little work is found reported in literature employing lipoamide reductase activity as an index of spoilage of fish.

Although many workers have reported that some of the dehydrogenases of carbohydrate metabolism could be employed for investigations related to the

post-mortem deterioration of fish quality, little has been reported on lactate dehydrogenase. Of the many enzymes studied much work has been reported on succinic dehydrogenase. A gradual disappearance of succinic dehydrogenase activity was observed during ice storage of fish. Fukuda (1957) has proposed the assay of succinic dehydrogenase for determining freshness of fish. Mochinga (1969) has correlated the degree of freshness of shucked oysters with the gradual disappearance of succinic dehydrogenase activity from the gill tissue. Frigerio et al (1980) have found that chilling of fish reduces this enzyme activity.

In a study of iced haddock, the reactivity of α glycerophosphate dehydrogenase to differing concentrations of magnesium ions added to the assay mixture was used as a measure of actual time elapsed from death and was interpreted as an indicator of leaching of the flesh (Gould, 1969). Reportedly a very sensitive gauge of ice storage age, it was accurate only to the first week of ice storage at $4^{o}C$. Because of the cellular disruption that occurs in frozen and thawed fish tissue, glycerophosphate dehydrogenase test as an index of leaching was not considered suitable for use with frozen stored fish.

Mitochondrial malate and glutamate dehydrogenases activities of muscle were found to increase during ice storage. Vana et al (1981) reported that in beef Psoas major muscle stored at 0 - $4^{o}C$ mitochondrial malate dehydrogenase and glutamate dehydrogenase activity increased with storage period.

LDH activities of different species of fish and shellfish have been reported. Lactate dehydrogenase (EC 1.1.1.27 L - lactate: NAD oxido reductase) catalyses the final step in muscle glycolysis, i.e. the reduction of pyruvate to lactate with the oxidation of NADH to NAD^{+}.

$$\text{L - Lactate} + NAD^{+} \rightleftharpoons \text{Pyruvate} + NADH$$

In post mortem fish muscle in the absence of citric acid cycle which is the aerobic pathway of glycogen degradation, pyruvate is alternately reduced to lactic acid anaerobically. In the LDH molecule there are two different poly peptide chains, in five different permutations. The distribution of these two poly peptide chains was dependent whether the extract originated in aerobic tissue such as heart (where the H_4 isozyme predominates) or in anaerobic tissue as in skeletal muscle (where the M_4 isozyme predominates).

In tuna and flounder skeletal muscle lactate dehydrogenases are quite similar. However, the heart enzymes are quite different in the two species. Cahn et al (1962) obtained data indicating that the heart and muscle LDH of sole and halibut are identical. They reported that flat fish are the only group of vertebrates for which identical types of LDH were found in heart and muscle in the adult stage.

Effect of storage at accelerated conditions on lobster muscle LDH has been reported earlier. Kaloustian et al (1969) has observed that when the crude extract of lobster muscle in Tris-Cl (0.01 M) EDTA (0.001 M, PH 7.6) were incubated at $37^{o}C$, LDH activity rapidly diminished as a function of time.

The role of cathepsin D which is a major lysosomal acid proteinase involved in the intracellulose protein degradation in altering the post-mortem textural attributes resulting in the accentuated formation of free amino acids, peptides and non protein nitrogen during storage has been recognised in recent years (Parrish et al, 1969., Reddi et al, 1972., Caldwell, 1970). Several attempts have been made to purify cathepsin from the skeletal muscle of fish such as tilapia (Doke et al, 1980); Salmon (Ting et al, 1968) and albacore (Grominger, 1964). Makinodan et al (1982) purified cathepsin D from carp muscle. Cathepsin D is physio-

logically considered to take part in intra cellular digestion of protein. Proteolytic activity in mackerel stored in ice has been correlated to the rapid metabolic rate of the fish muscle (Brackar 1956). However, the relation between fish muscle cathepsin D and proteolysis after the death of fish is uncertain. Its participation in autolysis is doubtful.

Fish muscle proteinases maximally active at acid and alkaline pH values have been often described. It is of considerable interest to note that a proteinase active at neutral pH is also present (Makinodan et al, 1979). It is yet to understand whether this neutral proteinase has any observable activity under commercial conditions of storage. Proteases and peptidases have been shown to be active at $5^{o}C$ and $37^{o}C$ in sterile muscle (Makinodan et al, 1980). Further more using radio labelled substrate it has been suggested that both cathepsins and alkaline proteases could contribute to proteolysis under normal post mortem pH (Lin et al, 1980). The loss of elasticity in kamabako has been attributed to the activity during processing of an alkaline protease in the muscle (Iwata et al, 1979). Makinodan et al (1980) suggested the existence of endo and exo peptidases in carp muscle during autolysis.

It was shown that storage at elevated temperatures enhanced the post-mortem ageing effect of muscle connective tissue and resulted in an increased release or depolymerisation of collagen components from collagen fibres. Lysosomal glycosidases may have an important function in collagenolysis by degrading the associated proteoglycan components of the tissue matrix and ground substance and improving the susceptibility of collagens to collagen degrading enzymes. On a study on the influence of post mortem time and incubation temperature on the release of lysosomal enzyme, it was found that high temperature storage ($37^{o}C$) caused greater release of lysosomal enzymes (Wu et al, 1981).

Davey & Gilbert (1976) studied the effect of temperature on muscle tenderisation and have observed that the maximum rate of tenderisation take place at 60°C. They postulated that the proteolytic enzyme cathepsin C may be responsible for the greater tenderisation effect at 60°C. However, at 60°C fish muscle paste showed lower gelstrength. This was caused by muscle alkaline protease activity. Alkaline protease has optimum activity at pH 8.0 & 65°C (Makinodan & Ikeda, 1969) which differs from the properties of cathepsins.

Some works have been reported on studies conducted on enzymes involved in the breakdown of ATP. Fish muscle ATP is decomposed principally in the following course (Tarr, 1966., Eskin et al, 1971).

$$ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow Inosine \rightarrow \begin{cases} \text{Hypoxanthine} + \text{Ribose} \\ \text{Hypoxanthine} + \text{Ribose} \end{cases}$$

$$1\ PO_4$$

Accumulation of IMP was observed in lobster (Dingle et al, 1968) shrimp (Suryanarayana et al, 1969) and crab (Sasano & Hirata, 1973). Unlike in fishes, AMP was found to accumulate in squid before conversion to IMP. (Langille & Gill, 1984). In the early stages after death the reaction IMP → Inosine → Hypoxanthine occurs more slowly than those in the sequence ATP → AMP → IMP. This leads to the accumulation of IMP in fish meat in the early stage after death.

AMP deaminase activity has been reported in fish muscle. The precise function of AMP deaminase (EC.3.5.4.6 AMP amino hydrolase) is not yet well known (Raffin, 1984). It catalyses the reaction,

$$AMP + H_2O \rightleftharpoons IMP + NH_3$$

Among the many physiological roles proposed to this enzyme, the assumption that AMP deaminase may stabilise the adenylate energy change seems to be well established (Chapman & Atkinson, 1973). Pathways for the degradation of adenine nucleotides in the invertebrates have not been clearly established. Arai (1966) found no IMP in certain invertebrates and demonstrated that in many species AMP is dephospharylated to adenosine which is then deaminated to inosine.

AMP deaminase is regarded as an allosteric enzyme activated by ATP and monovalent cations and some cases inhibited by GTP. Besides its function in the balance of the level of adenine nucleotide, which may itself be involved in control of glycolysis, the production of ammonia may enhance the activity of phospho fructo kinase both by direct activation and by a pH effect on inhibition.

Adenylic acid has been found in several animal tissues such as heart muscle, liver and kidney but the highest activities are often found in white skeletal muscle (Conway and Cooke, 1939). High enzyme activities are also measured in fish gill. (Raffin & Leray, 1980).

Activity determination of adenosine 5 monophosphate aminohydrolase was carried out in cod muscle (Dingle and Hines, 1967). It was found that activity of the enzyme in the extract decreased on post rigor. In the transition to post rigor the enzyme appeared to become associated with the myosin fraction.

The nucleotide degradation pathway in shrimp and stability of the enzymes catalysing the reactions involved have not been clearly established. Arai

(1966) observed two pathways for the breakdown of adenine nucleotides in Japanese prawn (Pandalus hypsinotus). One involves the direct deamination of adenosine monophosphate to inosine monophosphate while the second involves dephosphorylation of adenosine monophosphate to adenosine which is followed by deamination to inosine. Both these pathways will subsequently lead to hypoxanthine. Cheuk et al (1979) investigated the stability of AMP deaminase and adenosine deaminase during post mortem ice storage of pink (Penaeus deorarum) and brown shrimp (Penaeus cuztecus) caught in the Gulf of Mexico. AMP deaminase activity was lost rapidly during the early stages of ice storage and no activity could be detected after ten days for pink and sixteen days for brown shrimp. Even though there was a gradual loss in activity adenosine deaminase could be detected in both species through the entire storage period of 21 days. The authors suggested that the activity of adenosine deaminase & AMP deaminase could potentially be used as quality indices for fresh shrimp held on ice.

1.2 FROZEN STORAGE STUDIES

Many enzyme systems potentially useful as a probe to quality control are present in fish muscle or in the tissue fluid obtained by pressing or centrifuging the flesh. Freezing and thawing the flesh solubilises even more enzyme activity, previously latent (Gould and Peters, 1971). The properties of certain enzymes in the sarcoplasm have been reported to change measurably under conditions of frozen storage.

Nucleotides undergo a predictably rapid and considerable change after death. Their pattern of breakdown has been studied in fish muscle undergoing frozen storage (Jones & Murray, 1961., Jones, 1965., Tomlinson & Geiger, 1963). Rates and patterns of nucleotide degradation in frozen fish differ widely from those

in iced fish, as do the specific activities of the enzymes involved (Jones & Murray, 1961., Fraser et al, 1961). Only traces of labile phosphates were found in fish muscle after frozen storage at different temperatures (Heen, 1953) and the breakdown of nucleotides seemed to be an objective index for only the first five to six days of storage, depending on the storage temperature. There is also a difference among species, the degradation in carp and trout flesh frozen in liquid air and stored at - 8^{o}C was complete in 10 days (trout) to four weeks (carp) (Partman, 1961).

ATPase was one of the first objects of concentrated enzymic investigation, because of its intimate association with the contractile elements and functions of muscle. Connell (1960) reported that although no change in ATPase activity could be detected in fish stored at - 7^{o}C, a definite drop occured at - 14^{o}C and even at - 22^{o}C. The decrease in activity, which was slight, bore no apparent relation either to the rate of loss of solubility or to the development of toughness.

Partmann (1954) has shown that the activity of ATPase, though apparently accelerated below 0^{o}C was erratic and did not parallel freezing denaturation changes and was therefore not a suitable index for decomposition of fish either during iced or frozen storage. Saito and Hidaka (1955) and Sawant & Magor (1961) confirmed Partmann's findings.

The ATPase activity in the muscular extracts of fish is greater than in homogenates. On storage of extracts, homogenates and also fish of different species , Kangur (1977) observed that the ATPase activity decreases differently in the different species. However no significant changes in ATPase activity could be established season wise or species wise. Fukuda et al (1981) studied the dena-

turation of muscle during freezing and subsequent frozen storage of deep sea fish muscle in terms of myofibrillar ATPase activity and solubility of muscular proteins. ATPase activity was found high and the extent of denaturation was most effectively reduced by storage at - 40°C as compared to storage at other temperatures such as - 20°C or -30°C.

Frozen storage studies were carried out on eviscerated and gilled fresh mullet (Mugil cephalus) at - 20°C for 1 year (Jiang, 1977). It was found that the solubility and Ca^{2+} ATPase activity of salt soluble proteins during frozen storage at - 20°C decreased markedly.

Thermal stability of fish myofibril was influenced by the environmental temperature at which the species live. Arrhenius plots for ATPase activity in the presence of Ca^{2+} was non linear whereas in the absence of Ca^{2+} the plot was linear over the same temperature range (Seki et al, 1979). The parameters such as enthalpy of activation, the entropy of activation and the free energy of activation increased from cold water to warm water habitats. A positive correlation existed between the above parameters and the environmental temperature.

Enzymes of carbohydrate metabolism were often employed as analytical tool in assessing quality of fish. Studies carried out on trout gave positive correlation of succinic dehydrogenase activity with decrease in freshness (Frittoli & Ruggeri, 1968). This enzyme undergoes a reduction in activity when subjected to freezing and hence qualitative and quantitative succinic dehydrogenase methods are useful when performed simultaneously on a fresh sample of the same product to recognise fresh sea foods from frozen ones (Parisi et al, 1978). Cooling and freezing reduce but not completely destroy this enzyme

activity (Frigerio et al, 1980). Frittoli and Ruggeri (1968) reported that succinic dehydrogenase could be used to distinguish between fresh and frozen fish. Similarly freezing and thawing of rat mitochondria resulted in decrease in succinic hydrogenase activity (Ivanov, 1979).

Freezing and thawing of fish flesh has been reported to solubilise a labile, latent form of malate dehydrogenase causing an increase in specific activity (Gould, 1968). During a five month period of frozen storage, cod, pollock and dab showed a progressive decline in malate dehydrogenase activity when stored at - 7°C but no loss at all when stored at - 29°C (Gould, 1964). It was suggested that the relative lability of latent malate dehydrogenase might be turned to advantage in determining loss of freshness in frozen stored fish. On 3 months frozen storage of bovine muscle at - 20°C, more or less sharp reduction in total extractable activity of glutamate dehydrogenase was observed. Total activity of succinate dehydrogenase did not alter very much. Frozen storage caused no significant increase in the activity of glutamate dehydrogenase and succinate dehydrogenase in the muscle press juice (Hamm & El - Badawi, 1984). Ciani & Salerni (1965) have suggested that the dehydrogenase activity of the muscle tissue estimated using triphenyl tetrazolium chloride can be used as a method in distinguishing fresh fish from thawed frozen fish.

Aldolase which catalyses the break down of hexose phosphate into two triose phosphates is found in most fishes (Burt & Jones, 1961). In a study of the enzyme in cod & haddock, Connell (1966) found that some activity was lost during freezing and thawing and that the remaining activity gradually diminished during a 60 week period of storage at - 14°C. For all practical pur-

poses, the decline in aldolase activity appeared to be regular enough to serve as a possible objective test of frozen storage deterioration. There are two possible causes for decline in aldolase activity during storage at - 14°C, denaturation of the enzyme and progressive lack of availability of the enzyme in the homogenates. The first of these causes is the most obvious for it has been widely invoked to explain the behaviour of other proteins during the frozen storage of fish.

Lipase release from lysosomes of rainbow trout (Salmo gairdnerii) muscle subjected to low temperatures was studied by Geromel and Montgomery (1980). Storage on ice and fast and intermediate rates of freezing did not cause the release of acid lipase from the lysosomes. However, slow freezing and fluctuation of temperatures (-12 to - 35°C) of the frozen fillet resulted in appreciable release of acid lipase from lysosomes. During frozen storage most of the release of acid lipase from the lysosomal fraction occured within the first month of storage. Olley et al (1962) studied lipolytic activity during cold storage at 7° and 14°C in gadoid and related species, flat fishes and elasmobranchs. Phospholipase activity found to be negligible in the three elasmobranchs studied, but in all the other species phospho lipase was atleast as important as lipase in producing FFA. Lipase activity has been reported to bear no relationship to the fat content of fish.

In order to develop an analytical method for differentiating fresh fish fillets from frozen and thawed ones, Hamm & Masic (1971) measured the release, on freezing and thawing of mitochondrial form of glutamate oxaloacetate trans aminase in carp fillets. This attempt was unsuccessful because the mitochondria were already destroyed to a high degree with concomitant

release of GOT iso enzyme during normal storage of carp fillets in ice. Based on studies conducted on haddock and cod, Gould (1971) reported that it is possible to determine whether or not the flesh of certain food fish has been frozen and thawed by measuring changes in the properties of soluble malate dehydrogenase activity.

The process of freezing and thawing animal tissues causes a remarkable release of cytochrome oxidase from mitochondria. The activity of cytochrome oxidase in extract of tissues after freezing and thawing increases by fifteen times in chicken liver, 2.5 times in trout and four times in beef muscle as compared to extracts of unfrozen sample. (Barbagli & Crescenzi, 1981). These results suggest that the determination of the release of cytochrome oxidase can be used as a method to distinguish fresh animal tissues from those which have been frozen and thawed.

The process of freezing animal tissues leads to the disruption of cellular organelles like mitochondria, lysosomes etc. releasing into the cell sap the enzymes bound to these structures. Hamm & Kormandy (1969) studying on bovine and porcine muscle found that freezing and thawing causes a remarkable increase in glutamic oxalacetic transaminase (localised in mitochondria) activity in the muscle press juice. Chhatbar and Velankar (1977) observed that freezing and thawing of four species of tropical fish lead to an increase in the total activity of aspartate amino transferase in tissue fluid due to the release of bound form of mitochondrial enzyme.

Barbagli and Crescenzi (1981) reported that the process of freezing and thawing of animal tissues causes a remarkable release of cytochrome oxidase

from mitochondria. The activity of cytochrome C oxidase in extracts of tissues after freezing increases by 2.5 times in trout as compared to extracts in frozen sample. The authors suggested that the release of cytochrome C oxidase can be used as a method to distinguish fresh animal tissues from those which have been frozen and thawed.

Cattaneo et al (1982) reported that the specific activity of the lysosomal enzyme α-glucosidase in press juice from trouts increased significantly on freezing and thawing while no difference was noticed in the activity of β.N-acetyl glucosaminidase. A similar method to distinguish between fresh and thawed pork was reported (Demmer & Werkmeister 1985). Fresh and thawed frozen meat is identified from the ratio of β-hydroxy acyl CoA dehydrogenase activity of a partially disintegrated sample to the total extracted enzyme activity. However Hamm & Badawi (1984) observed that frozen storage of bovine muscle at - 20^{o}C caused no significant increase in the activity of mitochondrial aconitase, fumarase, glutamate dehydrogenase in the muscle press juice.

Literature survey has shown that many enzyme systems potentially useful to quality control are present in the tissue of fishes. Iced and frozen storage of fish and shellfish reported to cause substantial changes in the activity of many enzymes. However, little change or even increase, was observed in some enzymes. The present study was undertaken to screen some of the fish muscle enzymes for their potentiality as indices of freshness of tropical fish. Activity of the enzymes in press juice was also determined simultaneously in different species of fish/shellfish stored in ice and in frozen condition.

Four enzymes were chosen for the present study. They were Ca^{2+} ATPase and AMP deaminase, enzymes associated with the breakdown of ATP, lactate de-

hydrogenase, an enzyme of carbohydrate metabolism and lipoamide reductase. Ca^{2+} ATPase is an enzyme associated with the rapid disappearance of ATP in fish muscle on post mortem storage. Stability of this enzyme has been studied in different species of tropical fishes during ice and frozen storage. AMP deaminase is an enzyme which catalyses the deamination of adenosine monophosphate to inosine monophosphate.

In post-mortem fish muscle in the absence of citric acid cycle which is the aerobic path way of glycogen degradation, pyruvate is reduced to lactic acid anaerobically. Lactate dehydrogenase catalyses this final step in muscle glycolysis. Although many workers reported that some of the dehydrogenases of carbohydrate metabolism could be employed for investigations related to the post-mortem deterioration of fish quality, little has been reported on lactate dehydrogenase. Hence, this enzyme was chosen for the present study.

Little work has been carried out in the past employing lipoamide reductase as an index of spoilage of fish. The simple and rapid method of assay of this enzyme is a second reason for choosing it for the study reported in this thesis.

Several objective chemical tests were carried out simultaneously, along with enzyme assays to determine the degree of freshness of fish. Such tests carried out on fish muscle in the present study were, total bacterial count, pH, α amino nitrogen, total volatile nitrogen, trimethyl amine nitrogen, free fatty acid, peroxide value and thiobarbituric acid number and the subjective method of sensory evaluation.

Six species of tropical fish/shell fish were employed in the present study. Mainly fresh water and brackish water species of fish which are economically important

were chosen. The different species chosen are mrigal, mullet, pearlspot, milk fish, tilapia and P. indicus.

Mrigal (Cirrhinus mrigala (Ham.)) is one of the Indian major carps. A natural inhabitant of the Gangetic river system, mrigal contributes to a major share of the fisheries of this river system and the connected impoundments. A bottom feeding omnivore, mrigal subsists mainly on decomposing plants and animal matter, algae, detritus etc. In natural waters it is reported to grow to a length of about a meter attaining a weight of 8-10 kg. Because of the ease in producing the seed in controlled conditions and the good growth attained in culture ponds, it is probably the most widely cultivated fish species in India. Normal growth rate in culture ponds is one Kg/year.

Mullet (Liza parsia (Ham. Buch)) is one of the most desirable groups of fishes from the consumer point of view. They are hardy, low in food chains being herbivores and highly tolerant to salinity fluctuations. In culture ponds they accept supplementary feeds.

Pearlspot (Etroplus suratensis (Bl.)) is another popular table fish, particularly in Kerala. It is one of the best suited fish for brackish-water culture and can be acclimatised to fresh water. This is the only cultivated brackish water fish that breeds in confinement. In nature, it is reported to grow up to 30 cm reaching a wt of $1\frac{1}{2}$ kg. Although the growth rate in culture ponds is much less as compared to that in nature, because of the high consumer preference, it occupies an important place in brackish water culture.

The milk fish (Chanos chanos (FORSKAL)) is cultivated in brackish waters throughout the world particularly in South East Asia. It is cultured on a large

scale in Indonesia, Philippines and Taiwan. It is reported to reach a maximum size of 150 cm and 20 Kg in nature. In culture ponds usually a weight of 500-1000 g is attained in a year in normal stocking densities. In India it commands a place next only to the mullets. It is found to grow faster in culture ponds either in monoculture or in polyculture.

Tilapia (Oreochromis mossambicus (Peters)) a natural inhabitant of the rivers on the east-coast of Africa, is now well established in different parts of the world and is probably the most widely cultivated fish the world over. It is cultivated in various environments like fresh and brackish water ponds, paddy fields, sewage-fed ponds and reservoirs. It is an omnivore, very hardy and tolerant to a wide range of salinity. It breeds throughout the year. It is reported to attain a weight of 850 g under favourable conditions at the end of the first year. Production rate of 4980 Kg/ha/year has been reported from fresh water ponds, while 7000 kg/ha/year is reported from the culture in sewage water ponds. Tilapia has low commercial value as a fresh fish and is considered as a poor man's food. Several high protein foods such as salted dried tilapia, tilapia canned in tomato sauce, curry sauce or chilli sauce can be successfully prepared from tilapia and are well accepted.

About 62 species of prawns and shrimps of the family penaeidae are present in the Indian waters. Of these Penaeus indicus Milne Edw. is an important species which is commercially exploited. West-coast of India accounts for more than 85% of the total marine prawn landing. Frozen prawn is exported to countries such as Japan, USA, France and the quantity exported during 1986 was 89000 tonnes valuing Rs.370 crores. Because of its tremendous significance in the sea food export trade of this country, Penaeus indicus

was chosen as a representative of the marine species, for the study reported here.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 FISH AND SHELLFISH

Fresh water fish, mrigal (Cirrhinus mrigala) used for the experiments reported in this thesis were collected from the fresh water culture ponds maintained by the college of Fisheries, Panangad. Fishes were caught, iced immediately and were brought to the laboratory. Brackishwater fishes such as mullet (Liza parzia) pearlspot (Etroplus suratensis), milkfish (Chanos chanos) and tilapia (Oreochromis mossambicus) were harvested from the brackish water ponds maintained by the college of Fisheries, Panangad. The gears used for the collection of the above fishes were either cast nets or dragnets.

Prawns (Penaeus indicus) were also collected from the culture ponds and prawn filtration field near the College of Fisheries, Panangad. The gears used for the collection were cast nets, prawn filtration nets and dragnets. They were immediately iced and brought to the laboratory for the study.

2.1.2 CHEMICALS

ATP, NAD and NADH used for the work reported in this thesis were the products of SISCO Research Laboratories, Bombay. 5'AMP was procured from V.P. Chest Institute, University of Delhi, Delhi-2. Bovine serum albumin was the product of Sigma Chemical Co., USA. Folin-Ciocalteau reagent was from BDH (India). Sodium lactate was the product of Lobo-Chemie. 2,6 dichlorophenol indophenol and TBA were obtained from E. Merck, Darmstadt, West Germany. All other chemicals used were of analytical grade.

2.2 REAGENTS

2.2.1 SODIUM LACTATE, 0.5M

Lactic acid was diluted with an equal volume of water. 5N NaOH was added in 2.0 ml portions to the diluted solution until it is alkaline to an outside indicator. The solution was heated to 80^{O}C inorder to hydrolyse inner ester. The cautious addition of NaOH followed by heating was continued until the solution remained neutral. Diluted to 94 ml.

2.2.2 ANSA REAGENT

A mixture of 12g of sodium metabisulphite, 1.2g of sodium sulphite and 200mg of 1- amino 2- naphthol 4- sulphonic acid (ANSA) was well powdered and dissolved in 100 ml of distilled water. The reagent was stored in a brown bottle at 0-5^{O}C.

2.3 METHODS

2.3.1 ICE STORAGE STUDIES

Experiments in ice storage were carried out by mixing fish and ice in the proportion 1:1 and storing the lot in fish box. Reicing was carried out every day in order to replace the ice lost by melting.

2.3.2 FROZEN STORAGE STUDIES

Fish and shellfish were quick frozen at -40^{O}C and were stored at -20 $\pm$ 2^{O}C.

2.3.3. CA^{2+} ATP_{ase} ASSAY

Ca^{2+} ATPase activity was measured as described by Noguchi and Matsumoto

(1970). The assay mixture contained 1.00 ml of 0.24 M Tris-maleate buffer (pH 6.3), 1.6 ml of 1.7 M KCl, 0.4 ml of 10 mM $CaCl_2$, 2 ml of 2.2 mM ATP (Na salt). The final volume of the reaction mixture was made up to 6.0 ml with 0.6 M KCl. 0.05 ml of enzyme extract was added and incubated at 25°C for 5 minutes. The reaction was stopped by 1.00 ml of cold 50% TCA and the tubes were placed in ice. The inorganic phosphate liberated due to Ca^{2+} ATPase activity was measured by the Fiske & subbarow method (1925). A standard was prepared by dissolving 1.3613 g KH_2PO_4 in 1L water. A few drops of chloroform added. Diluted 1:10 so that 1 ml corresponds to 1 mole of Pi.

Ca^{2+} ATPase specific activity was calculated as micromoles of inorganic phosphorous/min/mg protein.

PREPARATION OF EXTRACT

White skeletal muscles of fish were used for the preparation of enzyme extract. Prawns were beheaded, peeled and deveined and the dressed meat used for the preparation of enzyme extract.

Extracts for enzyme assay were prepared as per the method described by Gilmour (1955) and modified as follows. 10-15 g of fish muscle was blended with 7 times volume of 0.6 M KCl containing 0.01 M $NaHCO_3$ adjusted to pH 8.0, in a waring blender for 1 min. The extract was transferred to a 250 ml beaker and placed in a refrigerator for 1 hr with occasional stirring. Centrifuged at 3000 rpm for 15 minutes. This supernatant was used for ATPase assay.

2.3.4 LIPOAMIDE REDUCTASE ASSAY

Substrate specificity of lipoamide reductase is fairly broad. Thus suitable hydrogen donors are dihydrolipoyl derivatives, NAD and oxidised pyridine nucleotide analogs, $K_3Fe(CN)_6$, methylene blue, 2,6 dichloro phenol indophenol. NADH - dichlorophenol indophenol reductase activity is known as diaphorase activity. Lipoamide reductase assay was carried out by the method described by Massey (1966).

Enzyme activity was measured spectrophotometrically at 600 nm at 25^{o} by following the reduction of the dye, 2,6 dichlorophenol indophenol. Each cuvette contained potassium phosphate buffer 0.3 M pH 7.2 0.66 ml, bovine serum albumin 2% (W/v) in $3X10^{-2}$ M EDTA 0.1 ml, 2,6 dichlorophenol indophenol 10^{-3} M 0.75 ml, NADH 10^{-2}M 0.06 ml, water and finally enzyme to a total volume of 3 ml. OD change is noted at 600 nm for 3 mins.

SPECIFIC ACTIVITY

One enzyme unit is defined as that amount which causes an initial corrected rate of change (ΔE600) of 1.00 per min. Specific activity is expressed as unit/mg protein.

EXTRACTION OF LIPOAMIDE REDUCTASE

Enzyme activity was extracted as per the method described by Massey (1966). Extraction was carried out at $0\text{-}5^{o}C$. Minced fish muscle was suspended in 10 times its volume of cold distilled water. Stirred for 5 minutes. The mince was allowed to settle and the supernatant solution decanted. Washing was repeated 3-4 times until the supernatant was only slightly coloured.

The mince was then transferred to a waring blender with 5 times (W/V) cold 0.02 M Na_2HPO_4 and the homogenate was centrifuged for 15 minutes at 1400 g. The supernatant was used for lipoamide reductase assay.

2.3.5 LACTATE DEHYDROGENASE ASSAY

The most convenient assay for lactate dehydrogenase is a spectro-photometric measurement of the rate of appearance of the band at 340 nm in NADH. The assay was carried out at pH 10, since the reaction produces one equivalent of acid. LDH assay was done as per the procedure described by Neilands (1955).

Assay mixture consisted of 0.1 M glycine - NaOH buffer 1.8 ml, 0.5 M Sodium lactate 0.1 ml and $2X10^{-2}$M NAD 0.1 ml. 0.02 ml enzyme solution was rapidly transferred to the reaction mixture. OD change at 340 nm was recorded as a function of time using a Hitachi model double beam Spectrophotometer.

The activity measurements are derived from the elapsed time for a ΔE 340 of 0.03, i.e., over the OD range 0.01 to 0.04. Assuming molecular extinction coefficient of 6.2 x 10^3 for NADH, the ΔE340 of 0.03 corresponds to the formation of 9.7 millimicromoles of NADH in the 2 ml test volume. Specific activity of LDH was calculated as NADH micromoles/min/mg protein.

EXTRACTION

10-15 g of fish muscle was blended with 5 times its weight of cold distilled water for 1 minute. It was then transferred to a 250 ml beaker.

Kept in a refrigerator with occasional stirring for 30 minutes. Extract centrifuged at 6000 rpm for 10 minutes and the supernatent was used for LDH assay.

2.3.6 ADENOSINE 5' MONOPHOSPHATE DEAMINASE ASSAY

AMP deaminase activity was measured spectrophotometrically based on the decrease of optical density at 265 nm (Lee, 1963). The reaction mixture consisted of $1.35X10^{-3}$ M 5' AMP in water 0.1 ml, 0.15 M succinate buffer 2 ml and water to final volume of 2.9 ml. Decrease in OD was recorded at 265 nm for 2 minutes.

Under assay condition the reaction is first order with respect to AMP and the initial velocity is directly proportional to enzyme concentration. 10 units is defined as that amount of enzyme which catalyses an optical density change from 0.55 to 0.4 in 1 min at 265 nm. Specific activity is expressed as units/mg protein.

EXTRACTION

Extracts for enzyme assay were prepared from fish and shellfish by a slightly modified method of Cheuk et al (1979). 10-15 g of the muscle tissue were blended in a waring blender with 5 parts of ice cold extraction buffer (0.3 M KCl, 0.09 M KH_2PO_4 and 0.06 M K_2HPO_4 adjusted to pH 6.5). After 1 min of homogenising, the mixture was transferred to a 250 ml beaker and stirred occasionally for 1 hr in a refrigerator. The precipitate which was shown to contain no deaminase activity was removed by centrifugation at 6000 rpm for 10 mins and the supernatant liquid was used for the enzyme assay.

2.3.7 pH

This was carried out on a 2:1 water-fish homogenate using an Elico Model L1-120 digital pH meter with a glass electrode.

2.3.8 TOTAL BACTERIAL COUNT

TBC was determined adapting the method of FDA for Aerobic Plate count and modified as under. 25 g of the flesh from which surface glaze scraped off were homogenised with 225 ml sterile saline. Serial dilutions were made and plated in duplicate on nutrient agar (fortified with 3% sodium chloride) by spread plate. The bacterial count was determined after incubation at $37^{o}C$.

2.3.9 ∝ AMINO NITROGEN

∝amino nitrogen was determined by the method of Pope & Stevens (1939). A trichloro acetic acid extract of the fish was prepared. ∝amino nitrogen in 5 ml of the TCA extract was determined based on the formation of soluble copper compound through the reaction between the amino acid and excess copper in the form of copper phosphate. The amount of copper taken into solution was determined iodimetrically, by titrating against 0.01 N sodium thio sulphate using starch as indicator. ∝ amino nitrogen was calculated as mg N_2/100 g fish.

2.3.10 TOTAL VOLATILE NITROGEN AND TRIMETHYL AMINE

A trichloro acetic acid extract of the fish was prepared. TVN and TMA values were determined by a procedure based on the micro diffusion method of Conway (Beatty & Gibbons 1937). 1 ml of TCA extract was taken in the inner chamber of the Conway's microdiffusion unit. TVN

was liberated by treating it with 1 ml saturated sodium carbonate solution. Liberated TVN was absorbed in 1 ml of N/50 H_2SO_4 taken in the inner chamber of the unit. From the amount of acid consumed, TVN content was calculated.

For the determination of TMA content 1 ml of formaldehyde was added to the TCA extract taken in the outer chamber of the Conway's micro-diffusion unit to prevent liberation of all the other amines (primary & secondary) and ammonia. The TMA was absorbed in N/50 H_2SO_4 and was estimated by titration. Both TVN and TMA content were calculated as mg N_2/100 g fish muscle.

2.3.11 FREE FATTY ACID

Lipids were extracted from the minced muscle blended with twice its weight of anhydrous sodium sulphate by the Bligh & Dyer method (1959) using methanol and chloroform. Free fatty acids in a portion of the extract was estimated by employing the method of Dyer & Morton (1956). In this method FFA was determined by titrating with 0.01 N N_aOH using phenolphthalein as indicator. Value expressed as % FFA as oleic acid.

2.3.12 PEROXIDE VALUE

Peroxide value was determined, on a portion of the extract prepared for the determination of FFA, by the method outlined by the AOAC (1980).

2.3.13 THIOBARBITURIC ACID NUMBER

The TBA values of 10 g muscle were determined in duplicate by the malonaldehyde distillation method of Tarladgris et al (1960). In this

method malonaldehyde content in a portion of the distillate was estimated based on its reaction with TBA reagent. The red colour developed was measured at 538 nm using a Hitachi model spectrophotometer. The value was expressed as TBA number (mg malonaldehyde per kg muscle).

2.3.14 SENSORY EVALUATION

An analytical panel consisting of seven judges all trained in differentiating appearance, odour, texture, flavour and overall acceptability of ice-stored and frozen fish/shellfish were employed in sensory evaluation. A numerical scoring system developed by Shewan et al (1953) for the sensory assessment of the spoilage of fish was used for this purpose. The following sensory characteristics of fish/shellfish such as general appearance, odour, texture, flavour and overall acceptability score were evaluated and seperate scores given. Maximum score under general appearance and texture was five and maximum score under flavour and overall acceptability was 10.

Cooking of fish/shellfish was carried out by placing the meat in boiling brine (2%) for 10 minutes. Cooled samples were presented to the judges.

2.3.15 DETERMINATION OF PROTEIN

Protein was estimated according to the method of Lowry (1951). A small quantity of protein was precipitated by adding an equal volume of 10% TCA. The precipitate was collected by centrifugation (15 minutes at 3000 rpm) and was dissolved in 0.1 N NaOH.

To 1 ml of the protein solution (containing 50-300 mg of protein) was added 5 ml of alkaline copper sulphate reagent, mixed well and allowed to stand

at room temperature for 10 minutes. To this was added 0.5 ml of diluted (1:2 in water) Folin-Ciocalteau reagent and mixed thoroughly. After 30 minutes, the optical density was measured at 500 nm in a spectrophotometer. The instrument was calibrated using bovine serum albumin as standard.

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CHAPTER 3

EFFECT OF ICE STORAGE ON ENZYME ACTIVITIES IN FISH AND SHELLFISH

3.1 RESULTS

3.1.1 CA^{2+} ATPase ACTIVITY IN THE MUSCLE OF FISH AND SHELLFISH SUBJECTED TO ICE STORAGE

Ca^{2+} ATPase activity was determined in fish muscle extract prepared using 0.6 M KCl containing 0.01 M $NaHCO_3$ adjusted to pH 8.0 .

Results of Ca^{2+} ATPase assays in the six species of fish and shellfish are shown in Table 1. Highest enzyme activity (specific activity, 0.21) was found in P. indicus and lowest activity (specific activity, 0.106) in pearlspot. In other species of fish, activity ranged in between the above two extreme values.

In order to determine the effect of ice storage on Ca^{2+} ATPase activity in fish muscle extract a study was conducted by storing six species of fish in ice and periodically determining Ca^{2+} ATPase activity. The results are shown in Table I.

Only in the species mullet, pearlspot, milkfish and tilapia a regular fall in enzyme activity was observed with storage period. In the case of mrigal, during the early days of storage, a gradual increase in enzyme activity was observed. This was followed by a rapid decrease in activity from day 12 of ice storage. Ca^{2+} ATPase activity increased with days of ice storage in P. indicus.

NOTE: Ca^{2+} ATPase specific activity is expressed as μ mole Pi/min/mg protein.

TABLE 1. Ca^{2+} ATPase activity in the muscle of six species of fish and shellfish. Enzyme assays were conducted on fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. Ca^{2+} ATPase specific activity is expressed as μ mole Pi/min/mg protein.

Storage period days	Ca^{2+} ATPase specific activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.140	0.125	0.106	0.107	0.130	0.210
3	0.150	0.120	0.101	0.110	0.128	0.305
6	0.162	0.110	0.090	0.096	0.122	0.370
9	0.190	0.109	0.076	0.072	0.115	0.331
12	0.171	0.108	0.060	0.062	0.092	0.460
15	0.150	0.098	0.055	0.051	0.990	0.560

3.1.2 CORRELATION BETWEEN CA^{2+} ATP_{ase} ACTIVITY AND STORAGE PERIOD

In order to study the nature of relationship between the two variables, namely storage period and Ca^{2+} ATPase activity Karl Pearson's correlation co-efficients have been determined. Correlation coefficients have been computed by the formula,

$$r = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{\sqrt{\left[N\Sigma X^2 - (\Sigma X)^2\right]\left[N\Sigma Y^2 - (\Sigma Y)^2\right]}}$$

where r is the Karl Pearson's linear correlation coefficient giving the degree of linear relationship between the two variables X and Y. N stands for the number of pairs of observations made on X and Y (Yamane, 1967).

The conventional levels used in interpreting statistical significance, viz. 5% (significant , $P \leq 0.05$) and 1% (highly significant, $P \leq 0.01$) levels are followed throughout the discussion.

Figure I shows the trend in increase or decrease in Ca^{2+} ATPase activity with respect to days of storage in ice. The fall in Ca^{2+} ATPase activity with period of storage observed in the case of mullet, pearlspot, milkfish and tilapia was found statistically significant at 1% level ($P \leq 0.01$). The increase in Ca^{2+} ATPase activity with days of ice storage observed in the case of mrigal was statistically not significant. However it can be seen from the Figure that in the case of P. indicus a statistically significant ($P \leq 0.01$ level) increase in Ca^{2+} ATPase activity was observed with time of storage.

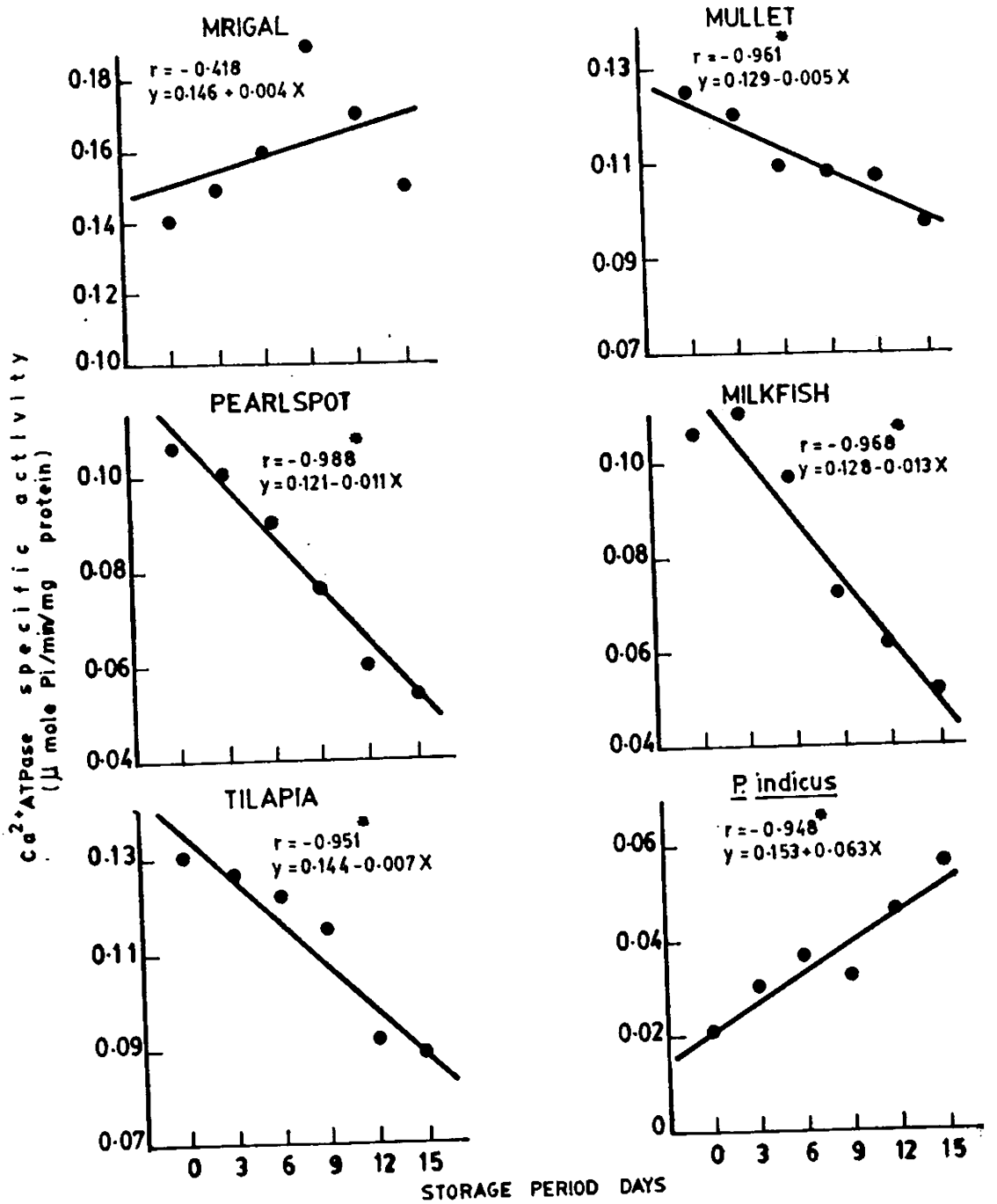

FIGURE 1. Stability of Ca^{2+} ATPase in the muscle of fish and shellfish subjected to storage in ice. Correlation coefficient (r) and regression lines showing the relationship between muscle Ca^{2+} ATPase activity and storage period are also given in the figure. Significance level,
* $P \leq 0.05$ (5% level)

3.1.3 CORRELATION OF CA^{2+} ATP_{ase} ACTIVITY WITH THE PHYSICO-CHEMICAL AND BIO-CHEMICAL INDICES OF SPOILAGE

Earlier studies have shown that physico-chemical and bio-chemical indices such as pH, total bacterial count, ∝amino nitrogen, total volatile nitrogen, free fatty acid, peroxide value, thiobarbituric acid number and sensory score can conveniently serve as indices of the degree of freshness of fish. Values of Ca^{2+} ATPase activity were statistically correlated with the values of the freshness tests performed at regular intervals during the period of ice storage. Correlation co-efficient of this relationship was determined and the results are shown in Table 2.

Inverse relationship was observed between Ca^{2+} ATPase activity and freshness indices such as pH, TBC, ∝amino nitrogen, TVN, FFA, PV and TBA number in most species of fish studied. That means with decrease in Ca^{2+} ATPase activity in these species during ice storage, freshness test values showed increase. When put to test for significance, it was observed that the negative correlation between enzyme activity and values of ∝amino nitrogen, TVN and PV were significant in four out of six species, studied. Although negative or positive correlation was observed in remaining two species of fishes, this was not found statistically significant. Similarly, although negative correlation were seen under the tests viz. pH, TBC, FFA and TBA number this was significant only in three species.

Sensory evaluation of the different species of fishes subjected to ice storage was carried out periodically. Overall acceptability score gradually decreased in all the samples subjected to ice storage. Significant positive correlation

TABLE 2. Correlation coefficient matrix between Ca^{2+} ATPase activity in fish muscle extract and freshness tests in the six species of fish and shellfish stored in ice. Significance level * $P \leq 0.05$, ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.390	-0.910**	-0.860*	-0.590	-0.860*	0.650
2.	TBC	0.310	-0.669	-0.930**	-0.735	-0.781	0.860*
3.	∝ amino N_2	0.001	-0.967**	-0.934**	-0.972**	-0.639	-0.830*
4.	TVN	0.246	-0.849*	-0.992**	-0.927**	-0.811*	-0.904*
5.	FFA	0.040	-0.880*	-0.940**	-0.930**	-0.730	0.640
6.	PV	0.289	-0.652	-0.915**	-0.935**	-0.887*	0.915**
7.	TBA number	0.638	-0.883*	-0.964**	-0.881*	-0.334	0.712
8.	Overall acceptability score	-0.418	0.871*	0.935**	0.982**	0.854*	-0.867*

was observed between Ca^{2+} ATPase activity and overall acceptability score in mullet, pearlspot, milkfish and tilapia. However in the case of P. indicus Ca^{2+} ATPase activity maintained an inverse relationship with overall acceptability score.

3.1.4 CA^{2+} ATPase ACTIVITY IN PRESS JUICE

Ca^{2+} ATPase activity in the press juice of fresh fish and shellfish were determined and the results shown in Table 3. Press juice exhibited lower Ca^{2+} ATPase activity than the muscle extract in all the species except mullet and tilapia. Lowest Ca^{2+} ATPase specific activity was found in pearlspot (specific activity 0.026). Substantial Ca^{2+} ATPase activity was found in mullet and tilapia. In these species the press juice ATPase specific activity was higher by 36% and 33.8% respectively than muscle extract activity. Highest Ca^{2+} ATPase activity was found in the press juice of tilapia (specific activity 0.174).

3.1.5 EFFECT OF ICE STORAGE ON PRESS JUICE CA^{2+} ATPase ACTIVITY

Results of periodic assay of Ca^{2+} ATPase in press juice of fish and shellfish stored in ice are shown in Table 3. Ice storage caused loss in press juice enzyme activity in all the species except milk fish and P. indicus. Considerable fall in enzyme activity with days of storage was observed in mullet, pearlspot and tilapia. In the case of mullet, specific activity decreased from the value 0.17 in fresh fish to the value 0.148 in fish at day 15 of storage. In the case of mrigal no significant fall in Ca^{2+} ATPase activity was observed with period of storage. During the initial days of storage press juice Ca^{2+}

TABLE 3. Ca^{2+} ATPase activity in the press juice of muscles of six species of fish and shellfish. Enzyme assays were conducted in the press juice of muscles of fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. Ca^{2+} ATPase specific activity is expressed as μmole Pi/min/mg protein.

Storage period, days	Ca^{2+} ATPase specific activity in press juice					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.042	0.170	0.026	0.083	0.174	0.140
3	0.041	0.169	0.025	0.089	0.162	0.151
6	0.038	0.160	0.038	0.077	0.151	0.189
9	0.046	0.158	0.021	0.091	0.138	0.198
12	0.032	0.150	0.010	0.095	0.122	0.210
15	0.041	0.148	0.015	0.098	0.115	0.265

NOTE: For the preparation of press juice 20 g of the white muscle from the middle and dorsal part of the body was taken, cut into pieces and centrifuged at $5^{\circ}C$ at 17000 rev. min^{-1} for 20 minutes.

ATPase activity in mrigal showed a decreasing trend which was reversed at the end of 9 days storage. Specific activity in mrigal at the end of 15 days storage was 0.041.

Ice storage caused a rise in press juice specific activity in the case of milkfish and P. indicus. Considerable rise in press juice enzyme activity was observed in prawn. Ca^{2+} ATPase activity in the press juice of fresh prawn was 0.140. At the end of 15 days storage in ice, press juice ATPase specific activity increased to 0.265.

3.1.6 CORRELATION BETWEEN PRESS JUICE CA^{2+} ATPase ACTIVITY AND STORAGE PERIOD

Regression lines of press juice Ca^{2+} ATPase activity on storage period in different species of fish and shellfish are shown in Fig. 2. Although negative correlation was obtained between the above two variables in most of the species, this was significant only in the case of mullet and tilapia. Among the other species only in P. indicus positive correlation was observed.

3.1.7 CORRELATION BETWEEN CA^{2+} ATPase ACTIVITY IN PRESS JUICE AND FRESHNESS TESTS

Many of the physico-chemical and bio-chemical tests such as pH, TBC, α-amino nitrogen content of muscle etc. have been earlier suggested as indices of spoilage of fresh fish. Table 4 shows the correlation between Ca^{2+} ATPase activity in press juice and other freshness parameters of fish muscle. It can be seen from the Table that only under the tests

TABLE 4. Correlation coefficient matrix between Ca^{2+} ATPase activity in press juice of fish muscles and freshness tests in six species of fish and shellfish stored in ice. Significance level; * $P \leq 0.05$ ** $P \leq 0.01$.

Sl. No.		Correlation coefficients (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.162	-0.982**	-0.6047	0.540	-0.940**	0.740
2.	TBC	-0.024	-0.840*	-0.806	0.667	-0.746	0.827*
3.	α amino nitrogen	0.294	-0.657	-0.587	0.725	-0.778	-0.852*
4.	TVN	-0.187	-0.908*	-0.667	0.752	-0.927**	-0.856*
5.	FFA	-0.38	-0.850*	-0.650	-0.690	-0.740	0.800
6.	PV	-0.349	-0.81*	-0.767	0.756	-0.950**	0.935**
7.	TBA	-0.477	-0.911*	-0.658	0.506	-0.171	0.596
8.	Overall acceptability score	0.273	0.913*	0.431	-0.666	0.900*	0.937**

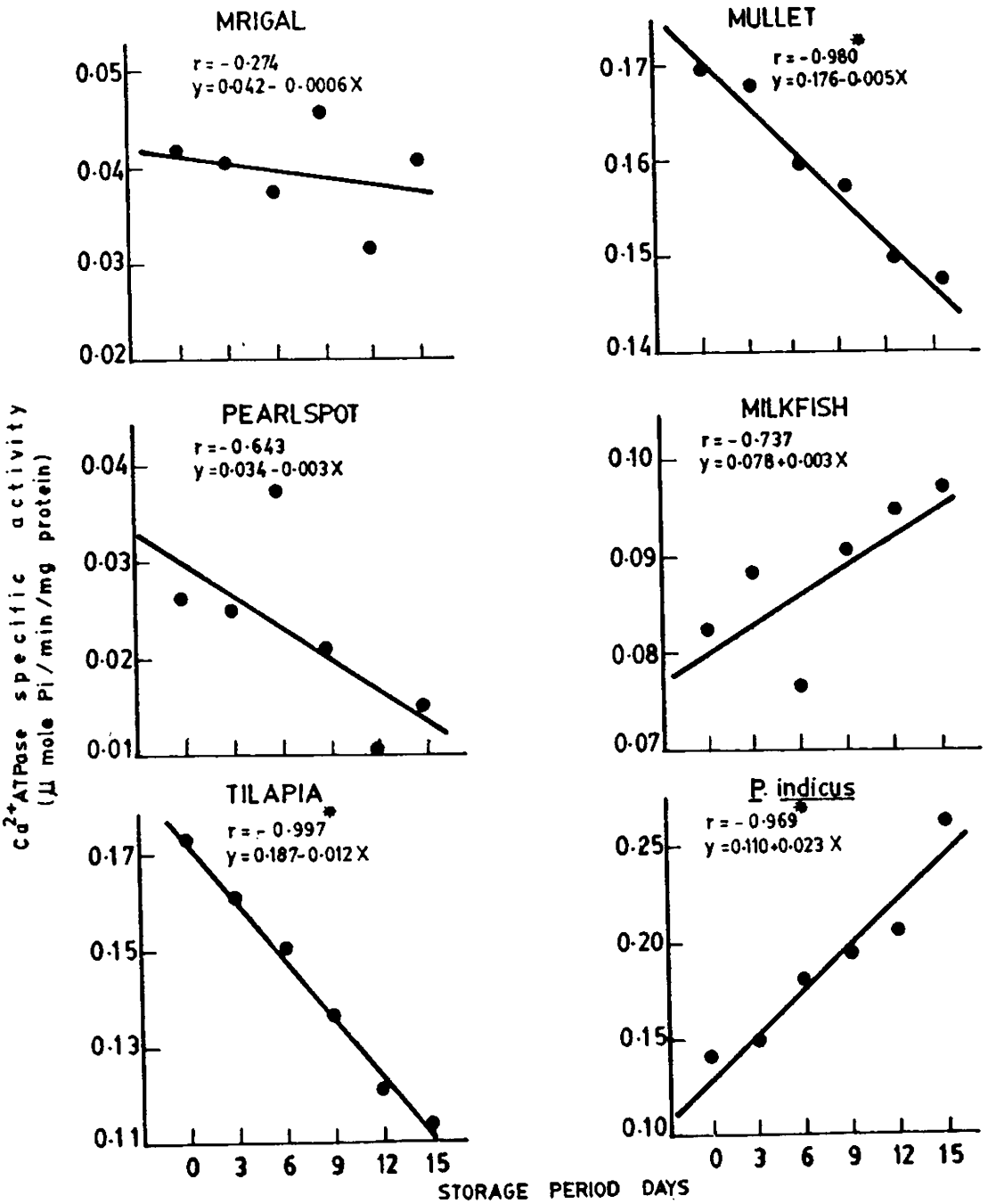

FIGURE 2 Stability of Ca^{2+} ATPase in press juice of muscles of fish and shellfish subjected to storage in ice. Correlation coefficients(r) and regression lines showing the relationship between pressjuice Ca^{2+} ATPase activity and storage period are also shown in the figure. Significance level, * $P \leq 0.05$ (5% level).

TVN and PV, negative correlation was observed in the species mullet, tilapia and prawn. Significant negative correlations between ATPase activity and freshness indices such as pH, TBC, α amino nitrogen, FFA and TBA number were observed only in a few species. Significant correlation was seen under the tests TBC, amino nitrogen, TVN, PV and overall acceptability score in the species P. indicus. Since only in a few species either negative or positive correlation was observed between Ca^{2+} ATPase activity and other freshness parameters, this enzyme activity in the press juice cannot be used as a reliable index of freshness of fish.

3.1.8 LIPOAMIDE REDUCTASE ACTIVITY IN FISH AND SHELLFISH MUSCLE

Lipoamide reductase activity in the muscle of six species of fish/shellfish were determined. Table 5 shows results of lipoamide reductase assay in various species of fish/shellfish. Substantial enzyme activity was observed in the fresh water species, mrigal, in which specific activity of the enzyme was 0.079 units/mg protein. Among the brackish water species studied, mullet showed the highest lipoamide reductase activity. In this species, specific activity of the enzyme was 0.08. Lipoamide reductase specific activity in P. indicus was 0.048.

3.1.9 CHANGES IN LIPOAMIDE REDUCTASE ACTIVITY IN FISH AND SHELLFISH SUBJECTED TO ICE STORAGE

Table 5 shows results of periodic determinations of lipoamide reductase activity of fish and shellfish subjected to ice storage for a period of 15 days.

NOTE: Lipoamide reductase specific activity is expressed as units/mg protein.

TABLE 5. Lipoamide reductase activity in the muscles of six species of fish and shellfish. Enzyme assays were conducted on fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. Lipoamide reductase specific activity is expressed as units/mg protein.

Storage period, days	Lipoamide reductase specific activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	*P. indicus*
0	0.079	0.080	0.039	0.047	0.056	0.048
3	0.110	0.070	0.038	0.036	0.058	0.034
6	0.118	0.062	0.038	0.026	0.060	0.021
9	0.080	0.058	0.036	0.020	0.060	0.021
12	0.085	0.052	0.037	0.018	0.062	0.013
15	0.093	0.052	0.030	0.016	0.063	0.013

A fall in enzyme activity was observed in mullet, pearlspot, milk fish and P. indicus. Among the above four species, P. indicus exhibited greatest fall in activity. Decrease in specific activity of the enzyme in the above species was by 0.035 units/mg protein. In the case of mullet, the fall in specific activity of the enzyme was only by 0.009. Contrary to the above observation, a steady increase in enzyme activity was observed in the two species mrigal and tilapia during the storage period of 15 days.

3.1.10 CORRELATION BETWEEN LIPOAMIDE REDUCTASE ACTIVITY IN FISH/SHELLFISH MUSCLE AND PERIOD OF ICE STORAGE

Fig. 3 shows correlation between lipoamide reductase activity versus time of ice storage in each of the six species of fish/shellfish studied. Negative correlation was found to exist between lipoamide reductase activity and time of ice storage in the species mrigal, mullet, pearlspot, milkfish and P. indicus. Significant ($P \leq 0.005$) fall in lipoamide reductase activity with time of ice storage was found in mullet, pearlspot, milkfish and prawn. Although negative correlation was observed in the fresh water species mrigal, this was not found statistically significant. Among the many species studied only in mullet and milk fish negative correlation significant at 1% level ($P \leq 0.01$) was observed.

3.1.11 CORRELATION BETWEEN LIPOAMIDE REDUCTASE ACTIVITY AND FRESHNESS TESTS

In each of the species, correlation between lipoamide reductase activity and freshness test values were determined and are shown in Table 6. Significant correlation under the tests pH & TBA number was found to exist in the species mullet and P. indicus. Although negative correlation was

TABLE 6. Correlation coefficient matrix between lipoamide reductase activity in fish muscle extract and freshness tests in six species of fish and shellfish stored in ice. Significance level: * $P \leq 0.05$ ** $P \leq 0.01$.

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	0.879*	-0.958**	-0.897*	-0.436	0.740	-0.867*
2.	TBC	-0.079	-0.776	-0.820*	-0.546	0.258	-0.681
3.	α amino nitrogen	-0.148	-0.984**	-0.912*	-0.964**	-0.146	0.978**
4.	TVN	-0.237	-0.800	-0.773	-0.888*	0.706	0.957**
5.	FFA	0.040	-0.738	-0.508	-0.93**	0.130	-0.611
6.	PV	-0.009	-0.704	-0.507	-0.884*	0.681	0.288
7.	TBA Number	-0.261	-0.861*	-0.871*	-0.949**	-0.238	-0.884*
8.	Overall acceptability score	0.141	0.942**	0.724	0.930**	0.761	0.917**

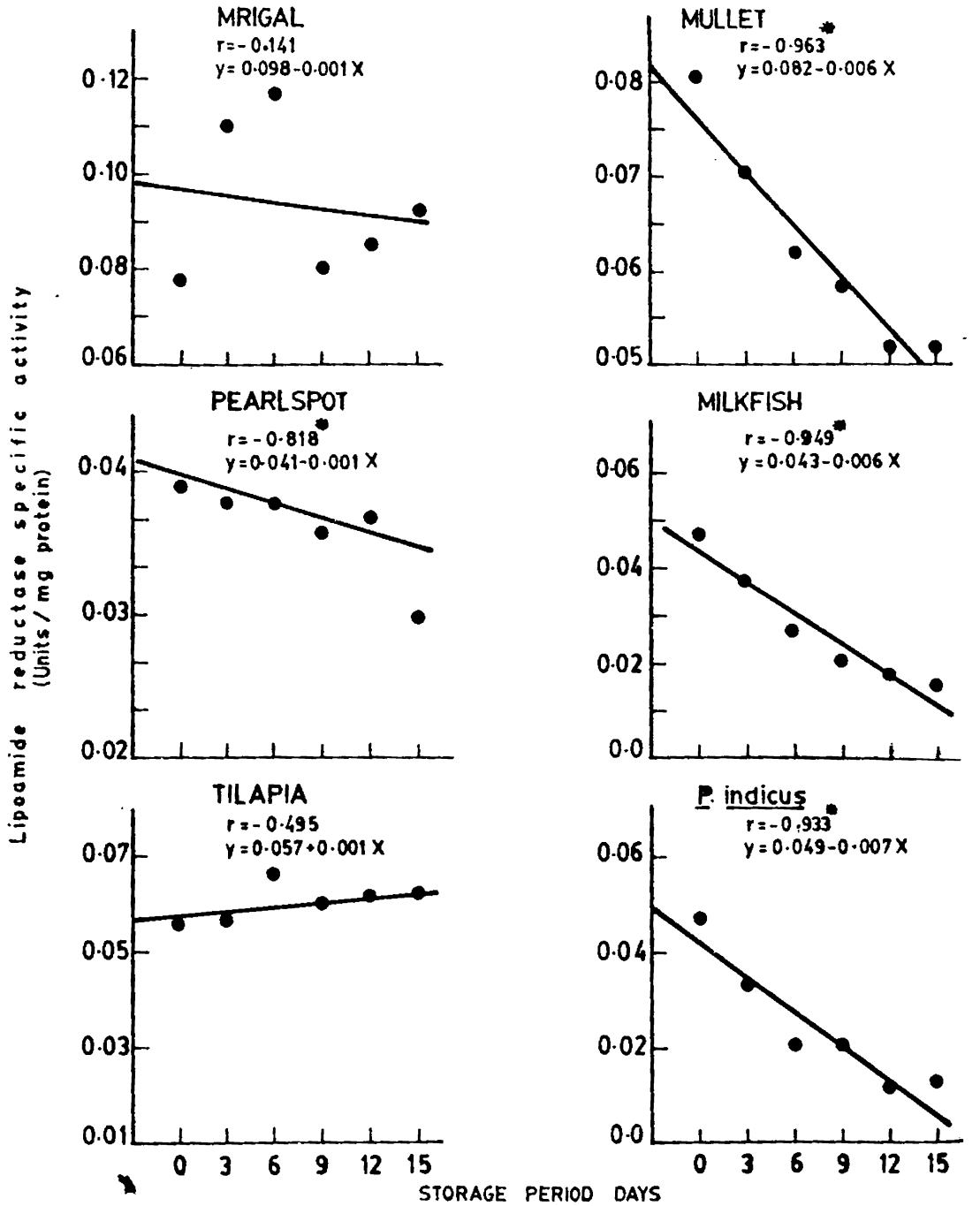

FIGURE 3. Stability of lipoamide reductive in the muscle of fish and shellfish subjected to storage in ice. Correlation coefficients and regression lines on muscle lipoamide reductase activity and storage period are shown in the figure. Significance level, * $P \leq 0.05$ (5% level).

seen between lipoamide reductase activity and other indices of fat spoilage such as FFA and peroxide value in four species this was significant only in milk fish. Significant positive correlation between lipoamide reductase activity and overall acceptability score has been observed in the species mullet and P. indicus.

3.1.12 LIPOAMIDE REDUCTASE ACTIVITY IN PRESS JUICE OF FISH MUSCLES

Table 7 shows lipoamide reductase activity in press juice of muscles of fresh fish and shellfish. Enzyme activity is lower in press juice than the muscle extract of all the six species of fresh water and brackish water fishes studied. P. indicus was an exception to this. Press juice lipoamide reductase activity in prawn was higher than that found in the muscle extract. Specific activity of the enzyme in the muscle extract was 0.048 whereas activity of the enzyme in press juice of P. indicus was 0.061.

3.1.13 EFFECT OF ICE STORAGE ON LIPOAMIDE REDUCTASE ACTIVITY

Results of periodic determinations of lipoamide reductase activity in the press juice of different species of fish and shellfish are tabulated in Table 7. It can be observed from the Table that ice storage caused an increase in specific activity of the enzyme in the species mullet, milk fish and tilapia. In tilapia, the increase in specific activity was by 0.025, the highest increase found when compared with all the other species. Ice storage resulted in a decrease in specific activity of the enzyme in the species mrigal, pearlspot and P. indicus. Among the above three species, greatest loss in enzyme activity was found in P. indicus. In this species loss in specific activity was 0.030 units/mg protein.

TABLE 7. Lipoamide reductase activity in the press juice of muscles of six species of fish and shellfish. Enzyme assays were made in the press juice of muscles of fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. Press juice was prepared as described in Table 3. Lipoamide reductase specific activity is expressed as units/mg protein.

Storage period, days	Lipoamide reductase specific activity in press juice					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
Fresh	0.040	0.044	0.026	0.017	0.016	0.061
3	0.041	0.046	0.024	0.019	0.020	0.067
6	0.043	0.049	0.018	0.014	0.030	0.058
9	0.039	0.047	0.016	0.021	0.029	0.048
12	0.036	0.050	0.014	0.026	0.036	0.040
15	0.032	0.051	0.016	0.036	0.041	0.031

3.1.14. CORRELATION BETWEEN ACTIVITY OF LIPOAMIDE REDUCTASE IN PRESS JUICE OF MUSCLE AND STORAGE PERIOD

The trend in increase or decrease of lipoamide reductase activity with period of ice storage was determined and the results are shown in Fig 4. Although negative correlation was found in the species mrigal, pearlspot and P. indicus only in the last two species mentioned this relationship was statistically significant ($P \leq 0.01$). Similarly, among the three species in which positive correlation was observed between lipoamide reductase activity and time of ice storage, this was found significant ($P \leq 0.05$) only in mullet and tilapia.

3.1.15 CORRELATION BETWEEN PRESSJUICE LIPOAMIDE REDUCTASE ACTIVITY AND FRESHNESS TESTS

Correlation coefficients between press juice lipoamide reductase activity and freshness tests for six species of fish and shellfish were worked out to establish the relationship between them. Results are shown in Table 8. These correlations were not uniformly significant within any one of the species under different freshness tests. Of the different freshness tests carried out FFA, PV and overall acceptability score alone gave significant correlation values with press juice lipoamide reductase activity. These values were significant in the species pearlspot, tilapia and P. indicus.

3.1.16 LACTATE DEHYDROGENASE ACTIVITY IN THE MUSCLE OF FRESH FISH AND SHELLFISH

Specific activity of lactate dehydrogenase in the muscle extracts of the fresh water species mrigal, brackish water species such as mullet, pearlspot, milkfish and tilapia and P. indicus were determined. The results are presented in Table 9. Among the different species of fish/shellfish studied, highest

TABLE 8. Correlation coefficient matrix between lipoamide reductase activity in press juice of muscles and freshness tests in six species of fish and shellfish stored in ice. Significance level; * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	0.710	0.860*	-0.720	0.800	0.980**	-0.660
2.	TBC	-0.874*	0.701	-0.706	0.919**	0.758	-0.913*
3.	∝ amino nitrogen	-0.795	0.907*	-0.759	0.797	0.689	0.766
4.	TVN	-0.924**	0.811*	-0.927**	0.896*	0.952**	0.762
5.	FFA	-0.820*	0.770	-0.960**	0.690	0.910*	-0.880*
6.	PV	-0.735	0.714	-0.849*	0.746	0.964**	-0.923**
7.	TBA number	-0.564	0.866*	-0.841*	0.681	0.153	-0.464
8.	Overall acceptability score	0.801	-0.764	0.951**	-0.794	-0.960**	0.917*

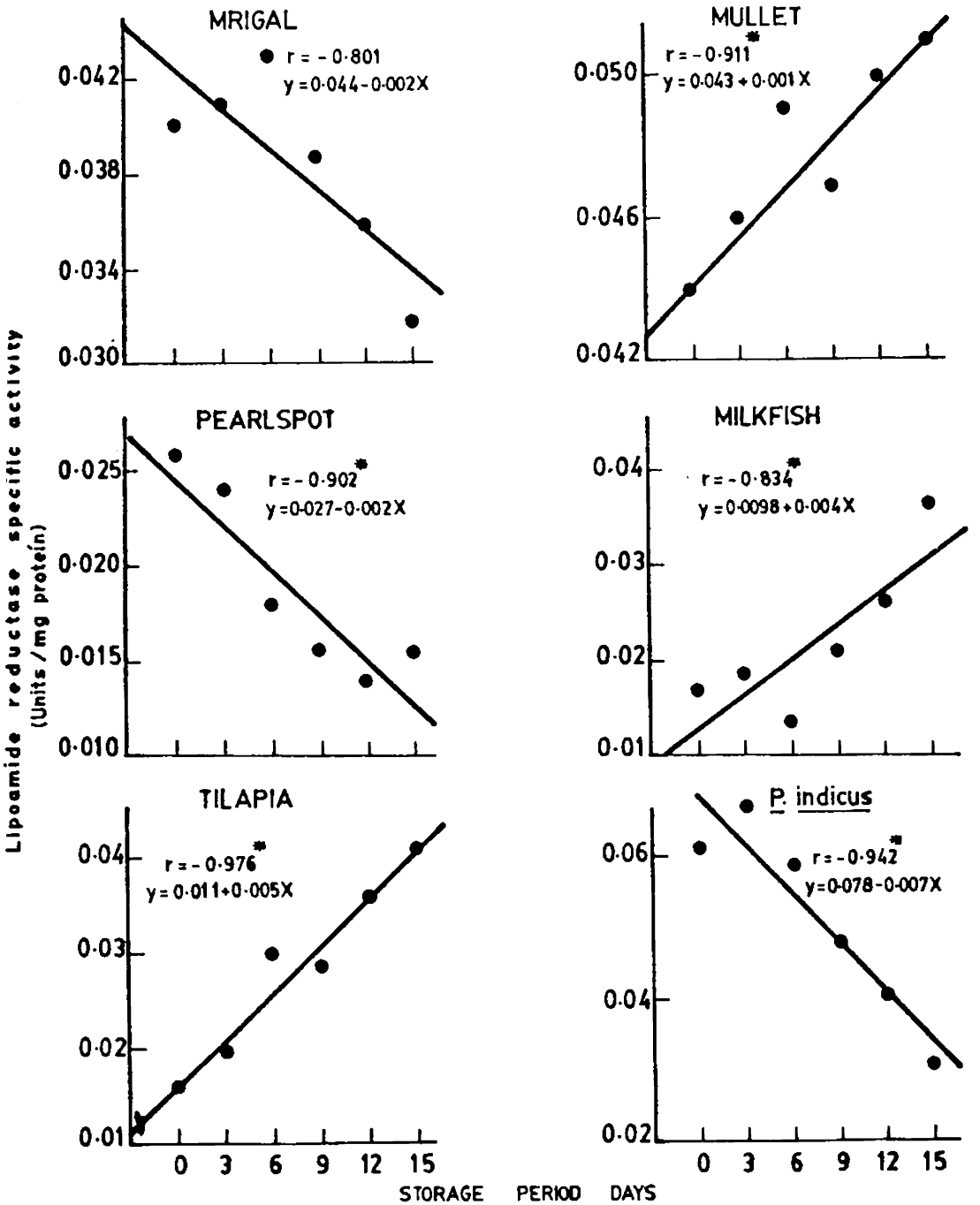

FIGURE 4. Stability of lipoamide reductase in the pressjuice of muscles of fish and shellfish subjected to storage in ice. Correlation coefficients and regression lines on press juice lipoamide reductase activity and storage period are shown in the figure. Significance level, * $P \leq 0.05$ (5% level)

LDH activity was observed in the brackish water species, milk fish. Mrigal also exhibited high LDH activity, next only to milk fish. Fresh mullet meat showed much lower LDH activity. Of all the species tested, lowest LDH activity was observed in P. indicus. Specific activity of the enzyme in fresh prawn muscle extract was 61.6, whereas the activity in fresh milk fish muscle was 936.5. This shows that specific activity of LDH in milk fish muscle is sixteen times higher than the activity of the enzyme, P. indicus.

3.1.17 EFFECT OF ICE STORAGE ON LACTATE DEHYDROGENASE ACTIVITY

Table 9 illustrates the changes in specific activity of LDH in the muscle extracts of different species of fish/shellfish stored in ice. It can be seen from the Table that ice storage caused pronounced fall in LDH activity in all the species studied.

Among the different species tested, specific activity of LDH in milk fish was found least affected by ice storage. Loss in specific activity of the enzyme was 0.9% of the initial activity at the end of 15 days ice storage. Greatest loss in LDH activity occured in P. indicus. At the end of 3 days ice storage, residual activity had fallen to 34.9% of the original activity. Prolonged storage resulted in further reduction in LDH activity. Residual activity in this species has fallen to 89% at the end of 12 days storage in ice. No LDH activity could be detected in P. indicus stored for 15 days in ice.

NOTE: LDH specific activity is defined as NADH μmoles/min/mg protein.

TABLE 9. LDH activity in the muscles of six species of fish and shellfish. Enzyme assays were made in fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. LDH specific activity is defined as NADH μmoles/min/mg protein.

Storage period, days	Lactate dehydrogenase specific activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	916.6	398.5	532.3	936.5	578.4	61.6
3	720.5	388.0	424.6	934.2	543.6	37.0
6	585.0	384.7	323.2	933.8	524.7	21.5
9	545.0	346.2	312.1	933.3	513.0	14.3
12	523.0	293.2	307.1	932.1	423.9	5.5
15	511.0	238.0	302.1	928.0	366.0	0.0

3.1.18 CORRELATION BETWEEN LACTATE DEHYDROGENASE ACTIVITY IN FISH/SHELLFISH MUSCLE AND PERIOD OF ICE STORAGE

The trend in increase or decrease in LDH activity with respect to days of storage has been studied and the results are shown in Fig. 5. Significant negative correlation was observed in all the samples studied. In the species mullet, tilapia and P. indicus, negative correlation was significant at 1% level ($P \leq 0.01$) and in the species mrigal, pearlspot and milkfish negative correlation was significant at 5% level ($P \leq 0.05$). This shows that significant loss of LDH activity takes place in fish and shellfish when subjected to ice storage.

3.1.19 CORRELATION BETWEEN LACTATE DEHYDROGENASE ACTIVITY AND FRESHNESS TESTS

Relationship between muscle LDH activity and freshness test values was studied. Karl Pearson's correlation coefficients were worked out as per the method described earlier and the results are shown in Table 10. Among the various indices tested overall acceptability score gives excellent positive correlation with muscle LDH activity. Similarly excellent correlation was seen under the tests TVN and TBA number. With the other tests although positive or negative correlations were observed this was significant only in a few species. In the case of mullet significant correlations were observed except with TBC test.

3.1.20 LACTATE DEHYDROGENASE ACTIVITY IN PRESS JUICE OF MUSCLE

LDH specific activity in press juice of muscle of fresh fish and shellfish were determined and the results are placed in Table 11. Lower LDH activity was observed in the press juice than muscle extract in all the species except P. indicus. In the press juice prepared from P. indicus, LDH specific activity was four times higher than the activity of the enzyme in muscle extract.

TABLE 10. Correlation coefficient matrix between LDH activity in muscle extract and freshness tests in the six species of fish and shellfish stored in ice. Significance level: * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficients (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.230	-0.930**	-0.690	-0.800	-0.850*	-0.890*
2.	TBC	-0.445	-0.797	-0.604	-0.910*	-0.897*	-0.705
3.	∝ amino nitrogen	-0.498	-0.857*	-0.740	-0.893*	-0.584	0.966**
4.	TVN	-0.764	-0.997**	-0.879*	-0.984**	-0.812*	0.959**
5.	FFA	-0.750	-0.960**	-0.860*	-0.760	-0.780	-0.630
6.	PV	-0.790	-0.909*	-0.742	-0.763	-0.859*	-0.526
7.	TBA number	-0.854*	-0.921**	-0.726	-0.883*	-0.457	-0.819*
8.	Overall acceptability score	0.899*	0.827*	0.941**	0.873*	0.823*	0.429

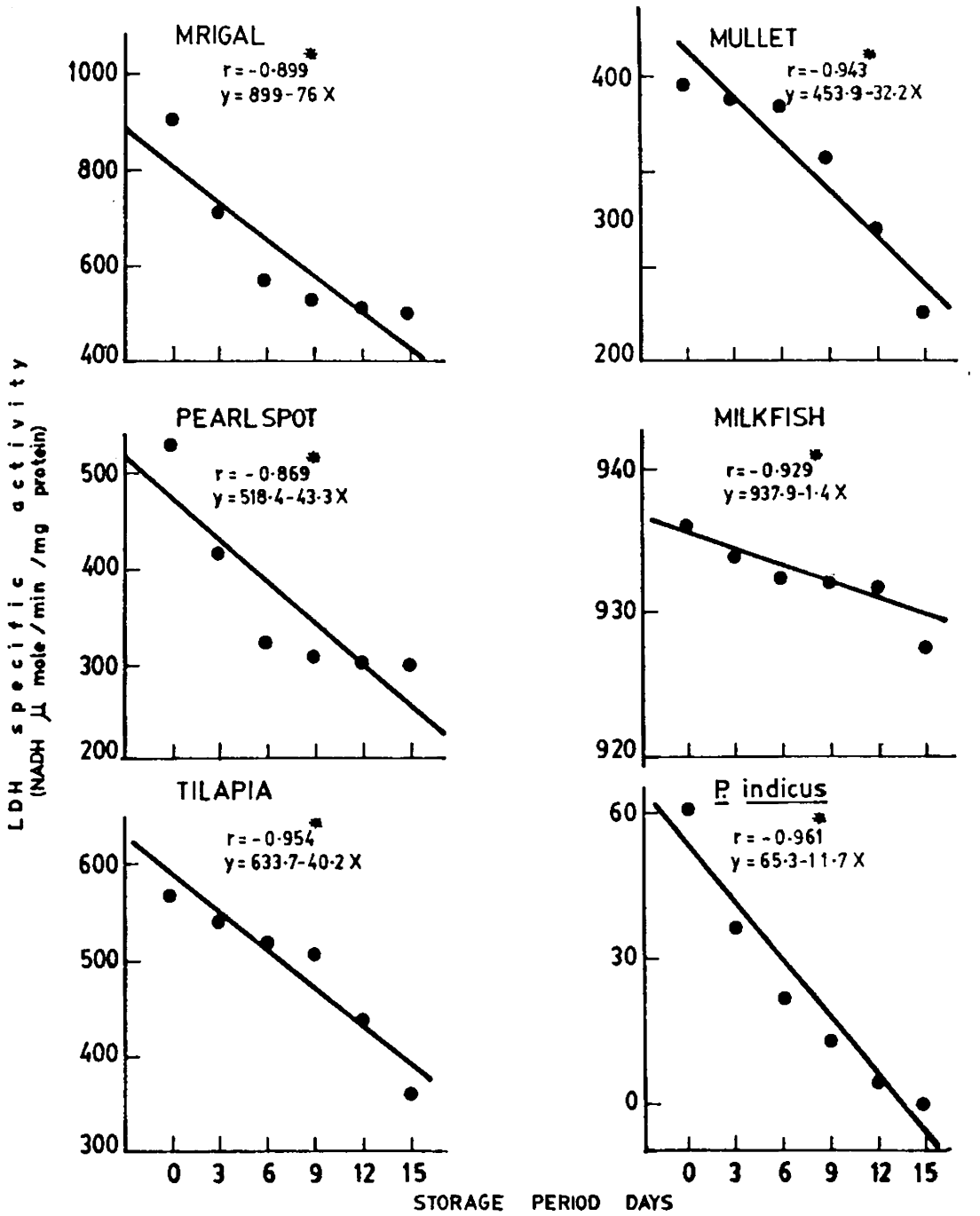

FIGURE 5 Stability of lactate dehydrogenase in the muscle of fish and shellfish subjected to storage in ice. Correlation coefficients and regression lines showing the relationship between muscle LDH activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

Among other species highest LDH activity was observed in mullet (specific activity, 287.8) and lowest enzyme activity was found in tilapia (specific activity, 139.5)

3.1.21 EFFECT OF ICE STORAGE ON PRESS JUICE LDH ACTIVITY

LDH activity in the press juice of muscle of fish and shellfish were determined at regular intervals and the results are placed in Table 11. Regular and progressive decrease in LDH activity was observed in all the species of fish and shellfish when subjected to ice storage. Greatest fall in enzyme activity was observed in P. indicus. At the end of 15 days storage in ice 97% loss in enzyme activity has occured. In the fresh water fish mrigal ice storage has caused little loss in enzyme activity.

3.1.22 CORRELATION BETWEEN PRESS JUICE LDH ACTIVITY AND TIME OF ICE STORAGE

Fig.6 shows correlation between LDH activity and days of storage in ice. Negative correlation has been observed in all the species studied. It shows that LDH activity decreases with days of storage in ice. The same pattern of correlation was observed between LDH activity in fish muscle extract and period of storage in ice.

Out of the six species studied, in mullet, pearlspot, milkfish and P. indicus significant negative correlation ($P \leq 0.01$) was seen between press juice LDH activity and time of ice storage. Correlation coefficient in the species mullet and pearlspot were 0.958 and 0.973 respectively. In the remaining two species, although negative correlation was observed between the above two variables, this was not found significant at 5% level ($P \leq 0.05$)

TABLE 11. LDH activity in the press juice of muscles of six species of fish and shellfish. Enzyme assays were made in the press juice of muscles of fresh fish and also in samples drawn on every 3rd day from the lot stored in ice. Press juice was prepared as described in Table 3. LDH specific activity is defined as NADH μmole/min/mg protein.

Storage period, days	Lactate dehydrogenase specific activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	142.1	287.8	231.2	261.4	139.5	244.2
3	146.8	286.1	184.6	255.8	124.7	148.2
6	138.5	248.9	123.0	164.5	118.0	84.4
9	122.4	233.1	112.6	122.3	113.0	46.5
12	118.5	226.0	96.5	111.2	109.8	20.3
15	128.4	218.0	48.7	107.6	117.5	6.2

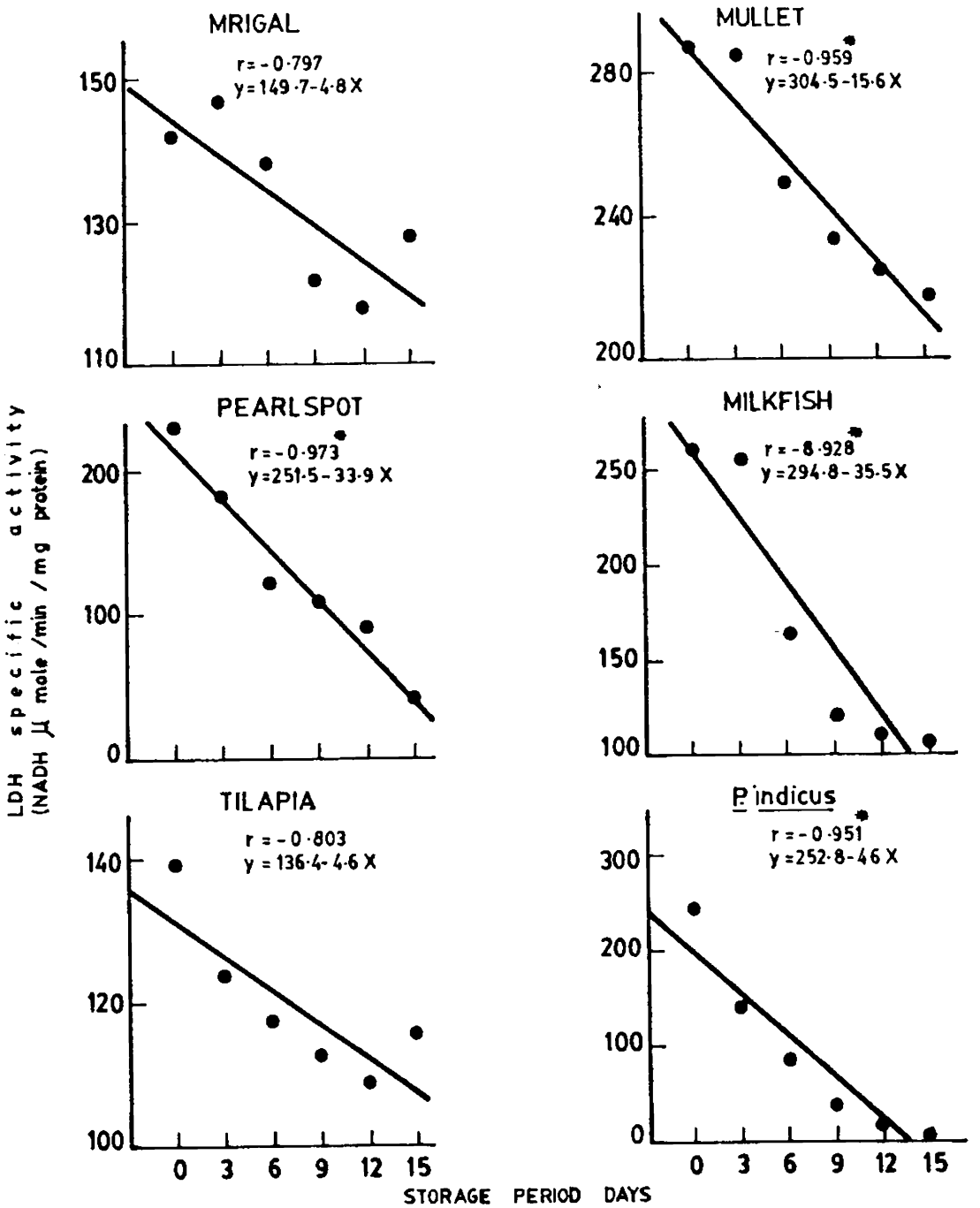

FIGURE 6. Stability of lactate dehydrogenase in the press juice of muscle of fish and shellfish subjected to ice storage. Correlation coefficients and regression lines showing the relationship between press juice LDH activity and storage period are also shown in the figure. Significance level

* $P \leq 0.05$ (5% level)

3.1.23 CORRELATION BETWEEN LDH ACTIVITY IN PRESS JUICE AND FRESHNESS TESTS

Karl Pearson's correlation coefficients between press juice LDH activity and freshness tests were determined and the results are placed in Table 12. Significant correlations were observed under the tests ∝amino nitrogen, TVN and over all acceptability score in most of the species studied. In the case of P. indicus muscle, positive correlation was observed with LDH activity and freshness tests such as ∝amino nitrogen and TVN. However negative correlations were yielded in the other species. Significant correlations under TBC test were yielded only in one species namely pearlspot. All the other tests also gave significant correlations in the same species.

3.1.24 5'AMP DEAMINASE ACTIVITY IN FRESH FISH AND SHELLFISH

5'AMP deaminase activity was determined in different species of fish and shellfish and the results are shown in Table 13. Higher enzyme activity was observed in tilapia and mullet muscle as compared to other species of fish. Activity of 5'AMP deaminase was very low in milk fish. Lowest enzyme activity was found in P. indicus in which case specific activity was 0.13 units/mg protein.

3.1.25 EFFECT OF ICE STORAGE OF FISH AND SHELLFISH ON 5'AMP DEAMINASE ACTIVITY

Table 13 shows results of 5' AMP deaminase assays conducted at the intervals of 3 days in different species of fish and shellfish stored in ice for a maximum period of 15 days. Ice storage resulted in loss in enzyme activity in all the species studied. In the case of milkfish 100% loss in enzyme activity was observed on storage for a period of 9 days. Ice storage of

TABLE 12. Correlation coefficient matrix between LDH activity in press juice of muscles and freshness test values in the six species of fish and shellfish stored in ice. Significance level * $P \leq 0.05$ ** $P \leq 0.01$.

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	*P. indicus*
1.	pH	-0.700	-0.960**	-0.840*	-0.149	-0.860*	-0.960**
2.	TBC	-0.293	-0.752	-0.813*	-0.534	-0.276	-0.675
3.	∝ amino nitrogen	-0.341	-0.951**	-0.927**	-0.957**	-0.782	0.972**
4.	TVN	-0.707	-0.820*	-0.963**	-0.849*	-0.916**	0.969**
5.	FFA	-0.550	-0.800	-0.880*	-0.939**	-0.670	0.630
6.	PV	-0.643	-0.791	-0.793	-0.848*	-0.409	-0.730
7.	TBA number	-0.943**	-0.808	-0.913*	-0.932**	0.315	-0.817*
8.	Overall acceptability score	0.796	0.953**	0.989**	0.962**	0.809	0.956**

TABLE 13. 5' AMP deaminase activity in the muscles of six species of fish and shellfish. Enzyme assays were made on fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. 5'AMP deaminase specific activity is expressed as units/mg protein.

Storage period, days	5' AMP deaminase specific activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	1.79	5.43	2.84	0.38	5.70	0.13
3	1.63	4.01	2.78	0.04	4.20	0.10
6	1.52	2.92	2.71	0.03	3.60	0.07
9	1.38	1.62	2.71	0.00	2.80	0.04
12	1.20	1.01	2.70	0.00	1.20	0.03
15	1.17	1.00	2.65	0.00	1.00	0.01

P. indicus caused rapid loss in deaminase activity. At the end of 15 days storage in ice, specific activity in P. indicus muscle extract has fallen to 0.11.

3.1.26 CORRELATION BETWEEN 5'AMP DEAMINASE ACTIVITY IN FISH AND PERIOD OF STORAGE IN ICE

The trend in increase or decrease in 5'AMP deaminase activity in relation to storage period was studied and the results are shown in Fig.7. Negative correlation significant at 1% level ($P \leq 0.01$) was observed between AMP deaminase activity in fish muscle and days of storage, in species such as mrigal, mullet, tilapia and P. indicus. Also in the species pearlspot, negative correlation was observed which was significant at 5% level ($P \leq 0.05$). The results indicate that 5' AMP deaminase activity in fish and shellfish significantly decrease with days of storage in ice.

3.1.27 CORRELATION BETWEEN 5'AMP DEAMINASE ACTIVITY AND FRESHNESS TEST VALUES

Karl Pearson's correlation coefficients between 5'AMP deaminase activity in fish muscle extract and freshness test values were determined at regular intervals during the period of ice storage in order to establish the relationship between them. The results are shown in Table 14. Significant correlations were observed under the test over all acceptability score in all the species studied except milkfish. Freshness tests such as α amino nitrogen and TVN values were having significant correlation with 5'AMP deaminase activity. It was observed that TBC test and PV did not give significant

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NOTE: 5'AMP deaminase specific activity is expressed as units/mg protein.

TABLE 14. Correlation coefficient matrix between 5'AMP deaminase activity in muscle extract and freshness test values in the six species of fish and shellfish stored in ice. Significance level. * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	0.420	-0.860*	-0.800	-0.26	-0.930**	-0.880*
2.	TBC	-0.651	-0.784	-0.751	-0.303	-0.707	-0.763
3.	∝amino nitrogen	-0.625	-0.983**	-0.892*	-0.731	-0.740	0.945**
4.	TVN	-0.479	-0.802	-0.933**	-0.685	-0.924**	0.946**
5.	FFA	-0.024	-0.750	-0.840	-0.700	-0.800	-0.740
6.	PV	-0.882*	-0.721	-0.753	-0.572	-0.963**	-0.832*
7.	TBA number	-0.909*	-0.824*	-0.883*	-0.753	-0.172	-0.726
8.	Overall acceptability score	0.990**	0.975**	0.982**	0.629	0.889*	0.989**

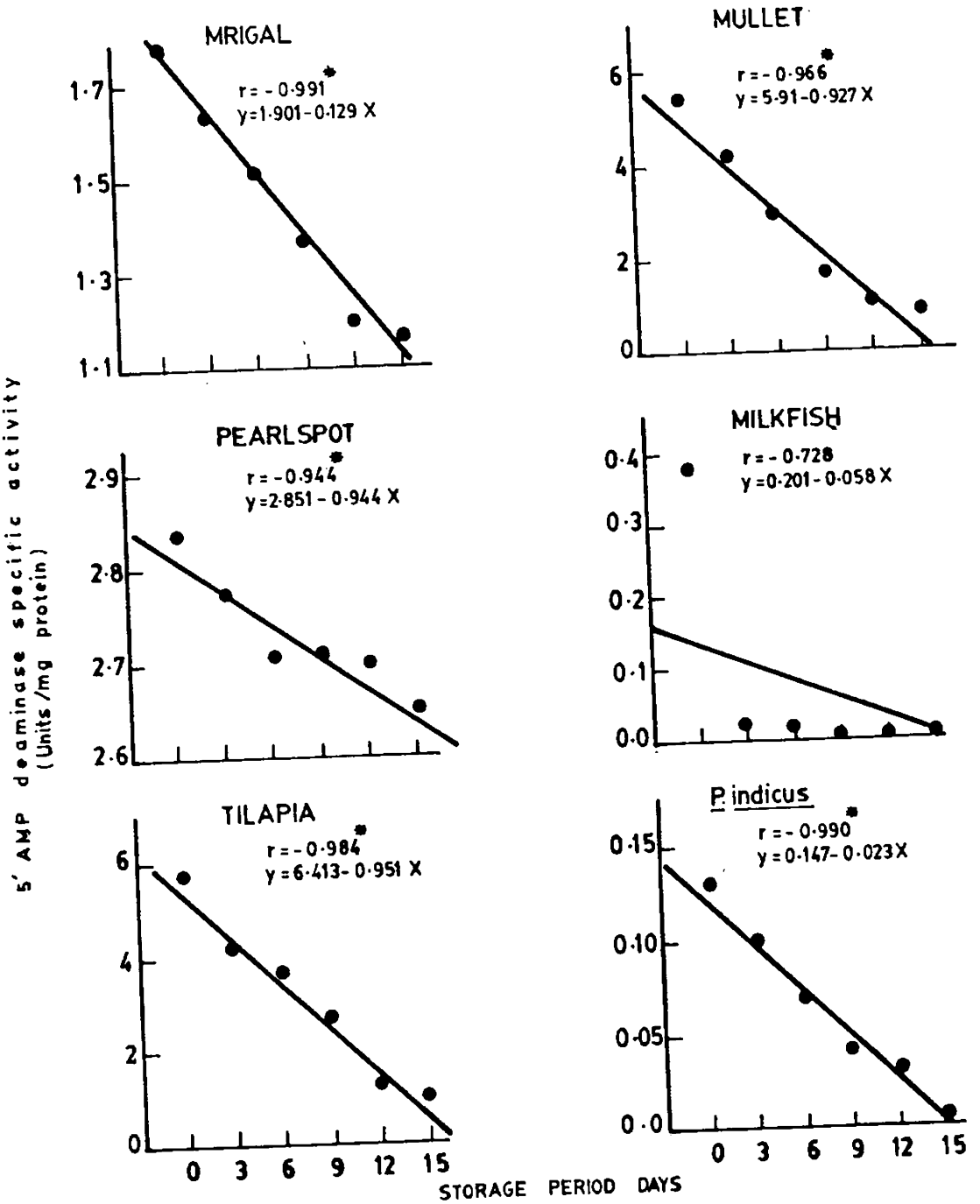

FIGURE 7. Stabiligy of 5' AMP deaminase in the muscle of fish and shellfish subjected to storage in ice. Correlation coefficients and regression lines showing the relationship between muscle 5' AMP deaminase activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

correlation in any one of the species studied. In P. indicus tests such as pH, α amino nitrogen, TVN, FFA and overall acceptability yielded significant correlations with enzyme activity.

3.1.28 5'AMP DEAMINASE ACTIVITY IN PRESS JUICE OF MUSCLES

Table 15 illustrates results of 5' AMP deaminase assays conducted in the press juice of muscles of fish and shell fish. Low deaminase activity was observed in the press juice than in muscle extract. The species pearlspot and P. indicus were exceptions to this. 5'AMP deaminase activity in the press juice of pearlspot and prawn were 24.6% and 42.3% higher respectively than the activity in muscle extract. Highest deaminase specific activity was observed in press juice of muscles of pearlspot (3.54) and lowest in mrigal (0.50).

3.1.29 EFFECT OF ICE STORAGE OF FISH AND SHELLFISH ON DEAMINASE ACTIVITY IN PRESS JUICE

5'AMP deaminase activity was determined in press juice of muscles of fish and shellfish at regular intervals during storage in ice. The results are shown in Table 15. It can be seen from the Table that ice storage of fish and shellfish caused fall in enzyme activity in all the species of fish/shellfish used for the study. In most of the species steady fall in enzyme activity was observed during the period of 15 days storage in ice.

Rapid fall in 5'AMP deaminase activity was observed in milkfish as a result of storage in ice. At the end of 3 days storage in ice, 50% loss in enzyme activity was observed. Fall in activity has gone up to 88% at the end of 6 days in ice. Similarly considerable fall in enzyme activity was observed in the press juice of pearlspot, tilapia and P. indicus.

TABLE 15. 5'AMP deaminase activity in the press juice of muscles of six species of fish and shellfish. Enzyme assays were conducted in the press juice of muscles of fresh fish and also on samples drawn on every 3rd day from the lot stored in ice. Press juice was prepared as described in Table 3. 5'AMP deaminase specific activity is expressed as units/mg protein.

Storage period, days	5' AMP deaminase activity					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.50	1.36	3.54	0.16	2.70	0.77
3	0.51	1.21	3.20	0.08	1.45	0.73
6	0.46	1.38	2.15	0.02	0.98	0.62
9	0.42	1.35	1.92	0.02	0.50	0.51
12	0.41	1.32	1.65	0.02	0.81	0.14
15	0.35	1.21	1.60	0.02	1.06	0.32

3.1.30 CORRELATION BETWEEN 5'AMP DEAMINASE ACTIVITY IN PRESS JUICE AND DAYS OF STORAGE IN ICE

Fig. 8 shows the trend in increase or decrease in 5' AMP deaminase activity in press juice with days of storage in ice. Negative correlation between the above two variables was observed in all the species studied. This shows that 5'AMP deaminase activity in press juice of muscle decreased with days of storage in ice. However correlation coefficients were significant only in mrigal, pearlspot and P. indicus. While in mrigal and pearlspot and the correlation coefficient was significant at 1% level ($P \leq 0.01$) in P. indicus it was significant at 5% level ($P \leq 0.05$).

3.1.31 CORRELATION BETWEEN 5'AMP DEAMINASE ACTIVITY IN PRESS JUICE OF MUSCLES AND FRESHNESS TESTS

Correlation coefficient values are shown in Table 16. In the species mrigal and mullet, highly significant correlations with press juice 5'AMP deaminase activity were observed under all the freshness tests except pH and TBC. Excellent correlations were observed between 5'AMP deaminase activity and TVN content in most of the species studied. Among the different freshness tests pH and TBC did not show significant correlation in any one of the species tested.

3.1.32 SENSORY EVALUATION OF FISH STORED IN ICE

The degree of freshness of fish was tested organoleptically. The following attributes were evaluated by a panel of judges.

TABLE 16. Correlation coefficient matrix between 5'AMP deaminase activity in press juice of muscles and freshness test values in the six species of fish and shellfish stored in ice. Significance level, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness indices	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	0.545	-0.180	-0.780	-0.300	-0.800	-0.640
2.	TBC	-0.800	-0.262	-0.757	-0.353	-0.220	-0.877*
3.	∝ amino nitrogen	-0.816*	-0.142	-0.839*	-0.837*	-0.802	0.780
4.	TVN	-0.947**	-0.469	-0.954**	-0.752	-0.887*	0.829*
5.	FFA	0.902*	-0.420	-0.940**	-0.800	-0.680	-0.660
6.	PV	0.935**	-0.332	-0.832*	-0.733	-0.826*	-0.766
7.	TBA number	-0.809	-0.379	-0.894*	-0.889*	0.408	-0.646
8.	Overall acceptability score	0.963**	-0.087	0.987**	0.786	0.721	0.858*

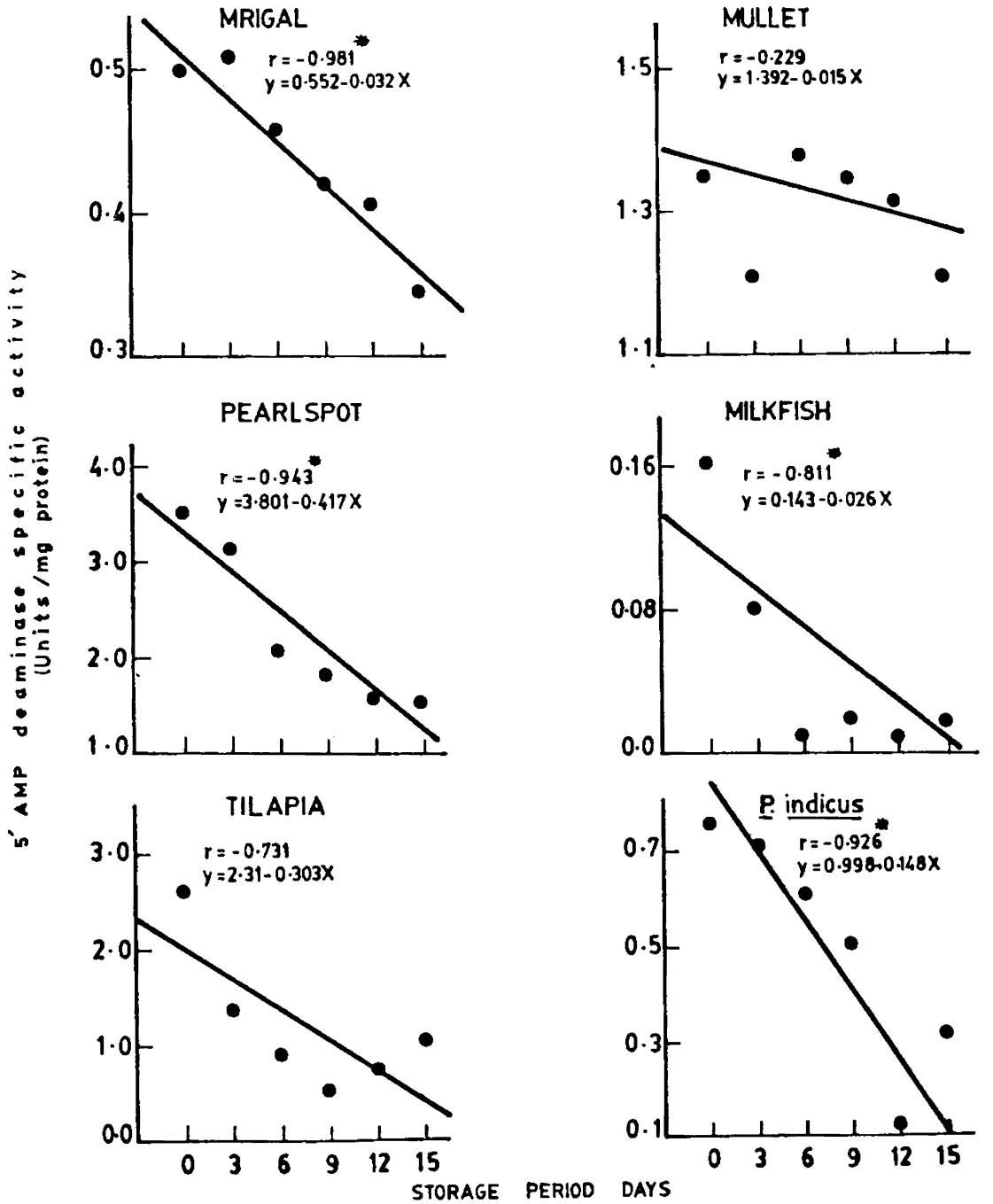

FIGURE 8. Stability of 5' AMP deaminase in the press juice of muscles of fish and shellfish subjected to storage in ice. Correlation coefficients and regression lines showing the relationship between LDH activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

Sl. No.	Attribute	Max score
Raw fish		
1.	General appearance	5
2.	Texture	5
Cooked fish		
3.	Odour	10
4.	Flavour	10
5.	Overall acceptability	10

3.1.33 GENERAL APPEARANCE

Table 17 shows results of evaluation of general appearance of fish and shellfish on a 5 point scale. Regular fall in general appearance score was observed in all the species studied. Karl Pearson's correlation coefficients were worked out to establish the relationship between general appearance score and storage period. Results are shown in Table 21. Negative correlation significant at 1% level ($P \leq 0.01$) was found in the species mullet, pearlspot and tilapia whereas negative correlation significant at 5% level ($P \leq 0.05$) was observed in the remaining species.

3.1.34 ODOUR SCORE

Odour of cooked samples were evaluated on a 10 point scale. 6 was taken as the score below which the sample was considered unacceptable. Results are shown in Table 18. Initial score of all the samples except mullet and pearlspot were 10. Mullet and pearlspot each showed an initial score of 9.

TABLE 17. General appearance scores of fish and shellfish stored in ice. Maximum score in the scale used was 5.

Storage period, days	General Appearance Score					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	5	5	5	5	5	4
3	3	4	4	3	4	3
6	3	3	2	3	3	1
9	3	1	2	2	2	1
12	1	1	1	2	2	1
15	0	0	1	1	1	0

TABLE 18. Effect of ice storage on cooked odour scores of fish and shell fish stored in ice. The scale ranged from 0-10.

Storage period, days	Cooked odour score					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	10	9	9	10	10	10
3	9	8	9	8	6	9
6	8	7	6	7	6	9
9	7	6	7	7	7	7
12	6	5	6	6	5	6
15	5	5	5	4	4	6

The trend in increase or decrease of odour score in relation to storage period was determined and the results placed in Table 21. Significant negative correlation was found between odour score and days of storage in the case of mrigal, mullet, pearlspot, milkfish and P. indicus. Although negative correlation was found in tilapia this was not found statistically significant.

3.1.35 TEXTURE EVALUATION

Texture of the samples has been evaluated and the results are shown in Table 19. Maximum texture score alloted was 5 in a scale ranging from 0-5. Ice storage caused reduction in texture score. Correlation coefficients were estimated in order to determine the correlation between texture score and days of storage. Results are shown in Table 21. Significant negative correlations ($P \leq 0.05$ level) between texture score and days of storage were found in mrigal, mullet, pearlspot, milkfish and P. indicus. Although negative correlation was found between texture score and days of storage in tilapia this was not found statistically significant.

3.1.36 FLAVOUR EVALUATION

Cooked meat of fish and shellfish were subjected to flavour evaluation by a trained panel of judges and the results shown in Table 20. Initial flavour score of the samples except pearlspot was 10 each. In the case of pearlspot initial score was 9.

Ice storage caused decrease in flavour score. Results of statistical evaluation of significance are shown in Table 21. Significant negative correlation ($P \leq 0.05$ level) was found between flavour score and days of storage in all the samples studied.

TABLE 19. Texture scores of fish and shellfish stored in ice. Texture scores were alloted to the samples based on a scale ranging from 0-5.

Storage period, days	Texture score					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	5	5	5	5	5	5
3	4	3	4	4	3	4
6	3	3	2	3	2	4
9	3	2	2	2	3	3
12	2	2	2	2	3	3
15	2	1	1	2	2	2

TABLE 20. Flavour scores of fish and shellfish stored in ice. Scale ranged from 0-10.

Storage period, days	Flavour score					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	10	10	9	10	10	10
3	9	8	9	9	8	8
6	7	8	8	8	7	8
9	7	5	6	6	5	7
12	6	5	5	5	5	6
15	4	5	4	4	4	6

3.1.37 OVERALL ACCEPTABILITY

Overall acceptability of the different samples were examined organoleptically and the results are shown in Table 22. Trend in increase or decrease in over all acceptability score against period of ice storage was determined and the results are shown in Table 21. Significant negative correlation ($P \leq 0.05$ level) was observed between overall all acceptability score and days of storage in mullet, pearlspot, milk fish, tilapia and *P. indicus.* Although negative correlation was found in mrigal, this was not found statistically significant.

3.1.38 CHANGES IN TBC OF FISH STORED IN ICE

Results of bacterial counts are shown in Table 23. Definite increase in total number of colonies per gm was observed with storage period in all the samples studied. The correlation coefficients between TBC and ice storage period were estimated. The results are shown in Table 28. Although increase in TBC was observed with storage period a linear relationship between the two was not observed. Absence of a significant linear relationship between the two variables indicates that TBC cannot be considered as a reliable index of freshness of fish.

The nature of relationship between TBC and other tests of freshness was determined by estimating Karl Pearlson's coefficients of linear correlations. Results are shown in Table 24. In majority of the cases these correlations were not significant though negative relations were dominating. Only TVN values gave significant results at least in three species. None of the correlation values were significant in the species *P. indicus.*

TABLE 21. Correlation coefficient matrix between storage period and organoleptic scores in the six species of fish and shell fish held in ice. Samples were withdrawn from the lot stored in ice on every 3rd day and sensory evaluation carried out. On every sample drawn, tests were conducted to determine score for various organoleptic characteristics; Correlation coefficients (r) between storage period and organoleptic scores were estimated as per the method given in the text. ** r significant at 1% level ($P \leq 0.01$) * r significant at 5% level ($P \leq 0.05$)

Sl. No.	Organoleptic Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	General appearance score	-0.941**	-0.979**	-0.943**	-0.938**	-0.980**	-0.923**
2.	Odour score	-0.941**	-0.982**	-0.894*	-0.962**	-0.828*	-0.962**
3.	Texture score	-0.971**	-0.939**	-0.923**	-0.930**	-0.683	-0.968**
4.	Flavour score	-0.962**	-0.925**	-0.976**	-0.994**	-0.970**	-0.951**
5.	Overall acceptability score	-0.999**	-0.938**	-0.960**	-0.983**	-0.899*	-0.979**

TABLE 22. Overall acceptability scores of fish and shellfish stored in ice. Overall acceptability scores were allotted to the samples by a panel of judges based on a scale ranging from 0-10.

Storage period, days	Overall acceptability score					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	*P. indicus*
0	9	10	10	9	9	9
3	8	9	9	9	9	8
6	7	8	6	7	5	7
9	6	5	6	6	6	5
12	5	5	5	5	4	5
15	4	5	4	4	4	4

TABLE 23. Effect of ice storage on Total Bacterial count of six species of fish and shell fish. Samples were with drawn periodically from the lot stored in ice and TBC determined.

Storage period, days	Total Bacterial count/g					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	5.5×10^3	2.1×10^3	2.6×10^3	2.9×10^3	2.9×10^3	1.06×10^3
2	9.9×10^3	0.7×10^3	0.2×10^3	6.4×10^3	6.8×10^3	0.86×10^3
5	2.5×10^4	1.2×10^4	1.3×10^4	1.3×10^4	3.2×10^4	1.80×10^3
8	3.0×10^4	8.7×10^4	2.6×10^5	3.1×10^5	6.0×10^4	2.8×10^4
11	6.2×10^5	3.3×10^5	8.4×10^5	6.2×10^5	2.5×10^5	3.8×10^6
14	3.9×10^6	1.8×10^5	1.1×10^6	4.2×10^6	1.1×10^6	4.4×10^6

3.1.39 pH DETERMINATIONS OF FISH AND SHELLFISH DURING STORAGE IN ICE

Muscle pH was studied in various species of fish and shellfish in fresh condition as well as during ice storage. Results are shown in Table 25. Muscle pH of various samples of fresh fish and shellfish was found distributed in the range 5.68 to 6.83. Lowest muscle pH was shown by milkfish and highest by P. indicus. The fresh water fish mrigal showed an initial pH of 6.29. Among the brackish water fishes, mullet showed highest initial pH (6.15). Tilapia and pearlspot muscle extract showed identical pH values. pH of prawn meat was near to neutral.

Results of muscle pH determinations during the period of ice storage of fish and shellfish are shown in Table 25. It can be seen from the Table that pH of fish muscle increased during storage in ice. All the species subjected to ice storage exhibited increase in pH. In the case of mrigal a steady increase in pH was observed till the end of 7th day. Thereafter, pH showed a rapid decrease. Further storage in ice caused only slight increase in muscle pH of mrigal.

Fresh prawn muscle showed pH near to neutral value. Ice storage of shellfish caused an increase in pH making it alkaline at the end of 3 days storage. Among the different species subjected to ice storage, highest increase in pH was observed in P. indicus.

Correlation coefficients were worked out to determine the trend in increase or decrease of pH with relation to storage period. The results are shown in Table 28. Significant positive correlations were found between pH and days of storage in mullet, pearlspot, tilapia and P. indicus.

TABLE 24. Correlation coefficients (r) between TBC and freshness parameters in the six species of fish and shellfish during storage in ice. n = 4 in each case. * Significant at the 5% level ($P \leq 0.05$) ** Significant at the 1% level ($P \leq 0.01$)

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.342	0.870*	0.797	0.831*	0.626	-0.313
2.	∝ amino nitrogen	0.597	0.699	0.942**	0.691	0.389	-0.577
3.	TVN	0.848*	0.797	0.901*	0.853*	-0.308	-0.723
4.	TMA	-	-	-	-	-	0.798

TABLE 25. Effect of ice storage on muscle pH of different species of fish and shellfish.

Storage period, days	pH					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	6.29	6.15	6.07	5.68	6.07	6.83
3	6.41	6.21	6.08	5.70	6.12	7.23
6	6.60	6.34	6.10	5.71	6.30	7.42
9	6.13	6.52	6.30	5.59	6.28	7.86
12	6.18	6.68	6.20	5.80	6.35	7.54
15	6.20	6.70	6.38	5.92	6.38	7.65

3.1.40 ∝ AMINO NITROGEN CONTENT IN FISH AND SHELLFISH DURING ICE STORAGE

Table 26 illustrates the results of ∝ amino nitrogen content determinations in fish and shellfish subjected to ice storage. ∝ amino nitrogen in the brackish water fishes, mullet, pearlspot and tilapia fall in the range 33.9 to 47.1 mg N_2/100 g sample. In the muscle of milk fish, comparatively higher ∝ amino nitrogen content was observed (108.7 mg N_2/100 g fish muscle). Among the various species studied highest ∝ amino nitrogen content was observed in P. indicus (240.1 mg N_2/100 g muscle).

Effect of ice storage on amino nitrogen in various fish and shellfish has been studied and results are shown in Table 26. A gradual increase in ∝ amino nitrogen was observed in all the species except P. indicus. Rise in ∝ amino nitrogen content was highest in tilapia subjected to ice storage. In this species an increase of 64 mg N_2/100 g, muscle occured at the end of 16 days storage in ice.

Remarkably regular fall in ∝ amino nitrogen content was observed in P. indicus stored in ice. At the end of 7 days storage in ice, ∝ amino nitrogen decreased by 159.5 mg nitrogen/100 g muscle. At the end of 16 days storage in ice, muscle ∝ amino nitrogen has decreased to 53.9 mg N/100 g muscle registering a decrease of 77.6% of the value obtained for fresh fish.

Correlation coefficients were worked out to determine the trend in increase or decrease of ∝ amino nitrogen content in muscle with days of storage. Results are shown in Table 28. In the species mullet, pearlspot and milkfish highly significant positive correlations were observed. In the case of P.indicus significant negative correlation ($P \leq 0.05$ level) was observed.

TABLE 26. Effect of ice storage on muscle∝amino nitrogen content in six species of fish and shellfish.

Storage period, days	∝ amino N_2, mg N_2/100 g muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	42.2	44.1	47.1	108.7	33.9	240.1
2	43.0	51.1	48.1	111.9	37.5	186.7
5	43.1	55.3	50.0	115.8	42.1	80.6
8	49.9	59.1	50.3	120.1	66.2	76.2
11	42.9	60.3	52.7	123.2	53.2	68.5
14	65.0	64.1	56.8	125.4	55.6	53.9

3.1.41 TRIMETHYL AMINE NITROGEN CONTENT IN SHELLFISH STORED IN ICE

Trimethyl amine content of iced P. indicus was determined at intervals of two days and the results are shown in Table 27. Fresh P. indicus showed TMA content of 0.48 mg N_2/100 g muscle. Ice storage caused a slight increase in TMA content. The trend in increase in TMA content with increasing storage period was studied. Although positive correlation was found between TMA content and days of storage in prawn meat, this was not found statistically significant (r = 0.751)

TABLE 27. Effect of ice storage on TMA content in P. indicus.

Storage period, days	0	4	7	10	13	16
TMA content, mg N_2/100 g muscle	0.48	0.52	0.42	0.60	0.71	1.82

3.1.42 DETERMINATIONS OF TOTAL VOLATILE NITROGEN IN FISH AND SHELLFISH SUBJECTED TO ICE STORAGE

Total volatile nitrogen content in the muscle of different species of fish and shellfish were determined and the data presented in Table 29. Among the various species studied, lowest TVN value was observed in pearlspot and highest in P. indicus. Initial TVN value of P. indicus muscle was 12.69 mg N_2/100 g muscle.

TABLE 28. Correlation coefficient matrix between storage period and values of freshness tests in six species of fish and shellfish held in ice. Correlation coefficients (r) between storage period and freshness test values were computed as per the method given in the text. ** r significant at 1% level ($P \leq 0.01$) * r significant at 5% level ($P \leq 0.05$)

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.397	0.983**	0.876*	0.650	0.940**	0.814*
2.	TBC	0.734	0.788	0.741	0.747	0.678	0.699
3.	∝ amino nitrogen	0.719	0.975**	0.954**	0.995**	0.779	- 0.900*
4	TVN	0.961**	0.923**	0.996**	0.978**	0.935**	- 0.932**
5.	FFA	0.918*	0.887*	0.907*	0.939*	0.877*	0.751
6.	PV	0.919*	0.854*	0.875*	0.929**	0.938**	0.895*
7.	TBA number	0.754	0.915*	0.961**	0.952**	0.179	0.675

TABLE 29. Effect of ice storage on total volatile nitrogen content in different species of fish and shellfish.

Storage period, days	Total volatile nitrogen, mg N/100g. muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	4.65	3.14	1.91	7.85	2.70	12.69
2	7.62	3.42	5.60	12.24	7.56	9.36
5	7.05	3.98	9.50	14.30	15.10	9.21
8	12.10	7.20	14.50	16.27	16.30	8.46
11	15.47	13.80	18.40	20.13	17.30	7.21
14	21.20	19.70	20.60	26.70	19.10	6.78

Effect of ice storage on TVN values were determined in different species of fish and shellfish. Steady increase in TVN value was observed in all the species subjected to ice storage except P. indicus.

The rise in TVN content in milkfish at the end of 16 days storage in ice was 18.9 mg N_2/100 g muscle which was highest among all the brackish water fishes studied.

On storage in ice TVN value in P. indicus showed a decrease and this gradual decrease continued till the end of storage period. Loss in TVN value at the end of 14 days storage was 5.91 mg N_2/ 100 g muscle.

Karl Pearson's correlation coefficients between storage period and TVN content are presented in Table 28. Significant positive correlations ($P \leq 0.05$ level) were found between TVN value and days of ice storage in all the species studied except P. indicus. In the case of P. indicus, significant negative correlation was found ($P \leq 0.05$ level).

3.1.43 DETERMINATION OF FREE FATTY ACID CONTENT

Free fatty acid contents of fresh as well as ice stored fish and shellfish were determined periodically and the results are shown in Table 30. In all the samples studied gradual increase in free fatty acid was observed. Among the different fishes subjected to ice storage, considerable rise in FFA was observed in tilapia and mullet. In prawn subjected to ice storage, least increase in FFA was observed.

Correlation coefficients, between storage period and FFA in muscle are shown in Table 28. In all the samples of fish subjected to ice storage, significant

Table 30. Effect of ice storage on free fatty acid content of six species of fish and shellfish.

Storage period, days	Free fatty acid, as % oleic acid					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.71	0.98	0.63	0.93	0.84	0.58
2	0.86	0.92	0.90	1.21	1.06	0.32
5	1.12	1.21	1.61	1.62	2.05	0.61
8	0.95	1.68	2.01	2.10	3.71	0.71
11	1.38	1.92	2.92	2.79	2.35	0.68
14	1.80	3.54	2.25	2.40	4.32	0.82

positive correlations ($P \leq 0.05$ level) were observed. Although positive correlation was found in prawn, this was not found statistically significant.

3.1.44 PEROXIDE VALUE DETERMINATIONS IN FISH/SHELLFISH STORED IN ICE

The results of peroxide value determinations are shown in Table 31. Among the different fishes studied, mullet showed highest initial peroxide content. Initial peroxide value of P. indicus meat was 0.42 milli equivalents/100 g muscle.

Ice storage of fish and shellfish caused an increase in PV of muscle. In most species studied, increase in peroxide value was gradual initially and more rapid towards the end of ice storage.

The data were subjected to statistical analysis in order to determine the correlation between PV and days of ice storage. The results are shown in Table 28. Significant positive correlation ($P \leq 0.05$ level) was found between PV and days of storage in mrigal, pearlspot, milkfish, tilapia and P. indicus. Although positive correlation was found in mullet, this was not statistically significant.

3.1.45 THIOBARBITURIC ACID NUMBER

The TBA number of fish muscle was determined in order to assess the extent of oxidative rancidity. Results are shown in Table 32. Low initial values were observed in all the samples. Ice storage of fish and shellfish caused slight increase in TBA number.

The trend in increase or decrease in TBA number with increasing days of storage was determined and the results are shown in Table 28. In all the

TABLE 31. Effect of ice storage on peroxide value in six species of fish and shell fish.

Storage period, days	Peroxide value, milli eq. Peroxide/100g fish muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.56	2.80	0.31	-	0.79	0.42
2	0.10	1.90	2.60	2.20	4.59	0.81
5	4.80	3.90	3.50	4.60	9.84	0.63
8	3.20	6.70	4.80	9.80	9.46	1.50
11	5.90	5.60	12.20	18.10	14.15	2.80
14	8.94	13.90	8.40	14.10	13.24	5.20

TABLE 32 Effect of ice storage on Thiobarbituric acid number in the muscle of six species of fish and shell fish.

Storage period, days	TBA number, mg malonaldehyde/100 g fish muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.093	0.610	0.210	0.050	0.042	0.021
2	0.085	0.720	0.200	0.210	0.045	0.032
5	0.468	0.780	0.280	0.610	0.021	0.046
8	0.628	0.710	0.320	0.520	0.012	0.035
11	0.936	0.920	0.410	0.720	0.036	0.044
14	0.638	1.010	0.520	0.880	0.061	0.039

species studied it was observed that TBA number increased with days of storage in ice. However this positive correlation between TBA number and days of storage was found significant ($P \leq 0.05$ level) only in mullet, pearlspot and milkfish.

...

3.2 DISCUSSION

Significant results were obtained from the studies on the stability of selected enzymes in fish and shellfish subjected to storage in ice. Rapid and considerable loss in the activities of the cytoplasmic enzymes, LDH and 5'AMP deaminase were observed in most of the species subjected to storage in ice. The steady decrease in the activities of these enzymes in ice stored fish can be developed as index of freshness of these food products.

The major problem in employing enzyme activity as an index of freshness of fish/shellfish is the variation in initial enzyme activity levels from species to species and also in different seasons. Before adopting any enzyme assay as index of freshness of ice stored fish, the test needs to be standardised based on the results of initial levels of enzyme activity.

An attempt has been made in this study to assign critical values to LDH and 5'AMP deaminase activities to indicate limits of acceptability. This has been carried out based on the correlation values obtained against sensory tests and other objective tests for freshness.

Consistent and significant correlations were not observed between the activities of the enzymes Ca^{2+} ATPase and lipoamide reductase and ice-storage period in the different species of fish and shellfish.

3.2.1 LACTATE DEHYDROGENASE ACTIVITY

Considerable LDH activity was found in all the species studied except P. indicus. Ice storage of fish and shellfish caused significant loss in muscle LDH activity. In the case of mullet, tilapia and P. indicus signi-

ficant negative correlation between LDH activity and time of storage was found. Similarly negative correlation significant at $P \leq 0.01$ level was found between LDH activity in press juice and days of ice storage in the species mullet, pearlspot, milkfish and P. indicus.

The following critical values were obtained for LDH activity in fish muscles stored in ice. Samples showing specific activities below these values will be considered unacceptable. These values were computed based on the corresponding values of sensory score and other freshness tests.

Fish/prawn	LDH activity (NADH μ mole/ min/mg protein)
Mrigal	545.0
Mullet	384.7
Pearlspot	312.1
Tilapia	513.0
Milkfish	933.3
P. indicus	21.5

The variation in the levels of initial activity has resulted in different limiting values for the species milkfish and P. indicus.

Similar findings were made by Rossmann & Liljas (1974) who have reported high specific activity (770 micromole min^{-1} mg^{-1} protein) of LDH in fish muscle. Janicke & Knopf (1968) observed high LDH specific activity in the anaerobic skeletal muscle than aerobic cardiac muscle in the case of

chicken and pig. Kaloustian et al (1969) showed that storage of lobster muscle at elevated storage temperature (37°C) caused rapid loss in LDH activity.

LDH activity in muscle extract and press juice of various species of fish and shellfish consistently maintained negative correlation with muscle pH during ice storage. In the case of mullet, maximum LDH activity was observed when pH was 6.15. Significant negative correlation was observed between LDH activity and pH in P. indicus. No LDH activity could be observed in prawn after ice storage for 15 days when the muscle pH was 7.65. Similar relationship between pH and LDH activity was observed in the species tilapia. Similarly significant negative correlation was observed between press juice LDH activity and pH in the species mullet, pearlspot, tilapia and P. indicus. Yamawaki & Tsukuda (1979) reported that the optimum pH at 30°C for gold fish liver LDH was 8.8. High enzyme activity at low values of muscle pH may be due to the maximum release of mitochondrial enzyme which takes place with the attainment of minimum muscle pH.

Negative correlation was observed between LDH activity in muscle extract and press juice and freshness parameters such as TBC, ∝amino nitrogen, TVN, FFA, PV and TBA number. Significant positive correlation was obtained between LDH activity in muscle extract and press juice and overall acceptability score. These results show that lactate dehydrogenase activity either in muscle extract or press juice can suitably be used as an index of freshness of fish and shellfish.

3.2.2 5'AMP DEAMINASE ACTIVITY

5'AMP deaminase activity in muscle extracts of most of the species tested suffered considerable loss when the fishes were stored in ice. Similarly loss in deaminase activity in press juice was observed in all the species studied. This trend was statistically significant in mrigal, pearlspot, milkfish and P. indicus. The above results agree to the one reported by Dingle & Hines (1967) on studies using cod muscle. In the transition to post rigor the enzyme appeared to become associated with the myosin fraction. Yeh et al (1978) reported that of the many ammonia producing enzymes, only AMP deaminase and adenosine deaminase were found to be present in significant level in white shrimp (P. setiferus). Similar to the results obtained in this study, Cheuk et al (1979) observed rapid loss of AMP deaminase activity during the early stage of ice storage and no activity could be detected after 10 days for pink shrimp and 16 days for brown shrimp.

Based on the results of sensory evaluation and other freshness tests, critical values of acceptability are assigned for 5'AMP deaminase activity in ice stored fish and shellfish. Ice stored fish and shellfish having lower 5'AMP deaminase activity are considered unacceptable.

Fish/prawn	5'AMP deaminase specific activity, units/mg protein
Mrigal	1.38
Mullet	2.92
Pearlspot	2.71
Tilapia	2.80
P. indicus	0.07

In the case of milkfish, specific activity has decreased to 0.03 at the end of 6 days storage in ice and at the end of 9 days storage total loss in activity occured.

Products formed as a result of degradation of adenine nucleotides particularly inosine monophosphate and hypoxanthine have been correlated with the quality and flavour of many species of fish (Tarr, 1966). The build up and subsequent decline of IMP in fish during storage has been attributed to a rapid deamination of adenosine 5' monophosphate to inosine monophosphate followed by a slow dephosphorylation of IMP to inosine.

White skeletal muscles of fish have often shown the highest AMP deaminase activity (Conway and Cooke, 1939). But Raffin and Leray (1980) have measured high enzyme activities in fish gill. Adenylic acid has been found in several animal tissues such as heart muscle, liver and kidney but the activities are lower than in skeletal muscle (Lee, 1957).

Muscle pH was found to have a significant negative correlation with 5'AMP deaminase activity in the muscle extract of mullet, tilapia and P. indicus during ice storage. However no significant relationship was observed between press juice AMP deaminase activity and muscle pH in any of the species studied. Similarly, it was observed by Yeh et al (1978) that the ammonia production by tissue enzymes is pH dependent with two optimal pH values of 6.0 and 8.5.

The present study has shown that activity of 5'AMP deaminase in fish muscle is negatively correlated with changes in freshness indices of the muscle, such as TBC, α amino nitrogen, TVN, FFA, PV and TBA number.

Positive correlation was found between muscle AMP deaminase activity and overall acceptability score. Similar trend was observed between changes in AMP deaminase activity in press juice and changes in freshness indices. However, in fewer number of species this relationship was found statistically significant.

3.2.3 CA^{2+} ATPase ACTIVITY

An initial increase in Ca^{2+} ATPase activity was observed in mullet, milkfish and tilapia subjected to ice storage. Enzymes bound to the membranes will be released on chilling fish and this can be the cause for initial rise in enzyme activity. The initial increase in enzyme activity was followed by steady decrease in activity. Considerable loss in enzyme activity has occured in mullet, pearlspot and tilapia subjected to ice storage for a period of 15 days.

Statistical correlation between Ca^{2+} ATPase activity and time of storage in ice was determined in all the species stored in ice. In four out of six species studied significant negative correlation was observed between Ca^{2+} ATPase activity in fish muscle and time of ice storage. Some what similar trend was observed between Ca^{2+} ATPase activity in press juice and days of storage in ice. In the case of P. indicus stored in ice, significant increase in Ca^{2+} ATPase activity in both muscle extract and press juice was observed on storage in ice for a period of 15 days.

Seki and Narita (1980) have studied the changes in ATPase activity of carp myofibrillar proteins, during ice storage and the results obtained in the present study agree to the findings of those workers. They report that

Ca^{2+} and Mg^{2+} ATPase activities gradually decrease during ice storage. Ca^{2+} ATPase activity in sardine myofibrillar proteins decreased to 50% of the initial levels after 6 days of ice storage (Seki et al, 1980). Higher initial values of ATPase activity in carp and tilapia were reported by Arai et al (1973). Specific activities reported were 0.35 and 0.356 in carp and tilapia respectively. Actomyosin Ca^{2+} ATPase activity of stone flounder and plaice changed little during 14 days ice storage (Ehira & Uchiyama, 1979). Ikeuchi et al (1980) reported that myofibrillar ATPase activity in the presence of Ca^{2+} ions decreased when rabbit muscle was stored at $37^{o}C$ for 0-12 hours.

ATPase activity in the muscular extracts of some fish species was found greater than in homogenates (Kangur, 1977). Similarly in the study reported here, greater Ca^{2+} ATPase activity was observed in the muscle extract than press juice in the species mrigal, pearlspot, milkfish and P. indicus. Changes in Ca^{2+} ATPase activity in fish muscle stored in ice negatively correlated with changes in pH of the muscle. Similar relationship was observed between Ca^{2+} ATPase activity in press juice and muscle pH. Seki et al (1979) reported that Ca^{2+} ATPase activity of carp myofibril stored in an alkaline medium increased but remained essentially unchanged at pH 6.1. The effect of pH on enzyme activity was determined between the range of pH 6.2 to 10.9 using two overlapping buffer systems. Both fresh water and sea water enzyme preparations showed optimum pH between 8.1 to 8.3 (Meitto & Chan, 1980).

Negative correlation was observed between changes in Ca^{2+} ATPase activity of fish muscle and changes in freshness indices such as TBC, α-amino

nitrogen, TVN, FFA, PV and TBA number. However since the results obtained were not consistent in all the six species studied, Ca^{2+} ATPase activity has little value in testing the freshness of fish/shellfish stored in ice.

3.2.4 LIPOAMIDE REDUCTASE ACTIVITY

In fish and shellfish low levels of lipoamide reductase activities were observed. Ice storage caused significant decrease in lipoamide reductase activity in some of the samples studied. In the case of press juice significant loss in lipoamide reductase activity with storage time was found only in pearlspot and P. indicus. In mullet and tilapia, ice storage caused significant increase in press juice activity.

Although negative correlation was observed between changes in fish muscle lipoamide reductase activity and freshness parameters such as TVN, FFA and PV in some of the species, this correlation was not significant in the remaining species. Lipoamide reductase being an enzyme of mitochondrial origin has not shown any promising result as index of freshness of ice-stored fish/shellfish.

3.2.5 CORRELATION BETWEEN ENZYME ACTIVITY AND SENSORY SCORE

Results of sensory evaluation of different fishes have shown good correlation between sensory score and period of storage. Significant negative correlation existed between general appearance score and days of storage in all the samples studied. Significant negative correlation was found between odour score and days of storage in mrigal, mullet, pearlspot milkfish and P. indicus. Similar trend was observed for texture scores also. Similarly

ice storage caused definite decrease in flavour score of the samples. Significant negative corelation was found between flavour score and time of storage in all the samples. Overall acceptability score of mullet, pearlspot, milk fish, tilapia and P. indicus were found to decrease significantly with days of storage.

Joseph et al (1980) while studying the ice storage characteristics of milkfish have reported similar findings. They have observed that fish were organoleptically in good condition for 4 days and in fair condition for 10-14 days but afterwards the eating quality deteriorated gradually due to toughening of texture and loss of juiciness and characteristic flavour. Sison et al (1982) reported that on ice storage fresh water fish deteriorate faster than brackish water fish.

In the study reported here no attempt was made to define the limit of acceptability because it was felt beyond the scope of a small panel. Nevertheless, the consensus among the panel members was that the sample became in edible when the overall acceptability score fell below 6.0. Assuming this to be the limit, given below are the shelf life of different fish and shellfish in ice storage.

No.	Sample	Shelf life, days
1.	Mrigal	9
2.	Mullet	6
3.	Pearlspot	9
4.	Milk fish	9
5.	Tilapia	9
6.	P. indicus	6

Varma et al (1983) reported that the shelf life of pearlspot, mullet and tilapia in ice were 12 days, 6 days and 11 days respectively. The reported storage period of marine fish in ice varied between 9 and 21 days (Bramsnaes, 1965). The overall keeping time of ungutted Patagonian hake (Merluccius hubbsi) in ice was found to be 15 days which can be compared well with that normally accepted for iced gutted cod. (Lupin et al 1980, Meyer et al 1969). The keeping time of offshore capelin (Mallotus villosus) stored in ice was 12 days. Reppond and Collins (1983) reported that based on sensory evaluation the quality of pacific cod was acceptable to 6 days. Curran et al (1980) evaluated sea bream in ice on a 10 point hedonic scale, 10 being good, 0 being bad and 4 being just unacceptable. Sea bream (R. sarba) remained in acceptable condition for up to 1 month at $0^{o}C$.

In the present study correlation between changes in enzyme activity in fish muscle and overall acceptability score was determined statistically. In most species studied significant positive correlation was observed between changes in LDH and AMP deaminase activities and overall acceptability score. Although positive correlation existed in the case of Ca^{2+} ATPase and lipoamide reductase activities, this was found significant only in a few species. Steady loss in LDH and AMP deaminase activity in fish stored in ice appears as reliable indices of the lowering of freshness as indicated by loss in overall acceptability score.

3.2.6 ENZYME ACTIVITY AND TOTAL BACTERIAL COUNT

Total bacterial count of the fishes increased during ice storage. However no linear relationship could be established between total bacterial count and storage period. Varma et al (1983) reported that total bacterial count

of iced pearlspot, mullet and tilapia increased with period of storage. They have observed that although TBC at incubation temperature of 28-30^{o}C increased with time of storage, at the incubation temperature of 37^{o}C the rise was not significant. This indicates that TBC is inapplicable as a quality index for iced fish. An increase in TBC with time of storage was reported earlier in the case of mrigal subjected to ice storage (Nair et al., 1971). Curran et al (1980) reports that total plate count of sea bream subjected to ice storage increases with time of storage. Similarly it was observed that holding sardine in ice for nine days resulted in an approximate 3 fold increase in their bacterial load. Similar observations were made by Shenoy & James (1972) on fresh water and brackish water tilapia. Joseph et al (1980) report that TBC of cultured chanos stored in ice rapidly increased till day 10 and further storage upto 18 days caused decrease in TBC. Garg and Stephen (1982) report that TBC of ice stored kati (pellona sp.) undergo decrease initially till day 9 and the count increases on prolonged storage.

No consistent relationship was observed between changes in enzyme activities and total bacterial count in the study reported in this thesis. Although in most cases negative correlation was found between changes in enzyme activities and TBC this was significant only in a few species. Bacterial growth is mainly responsible for the inconsistent pattern of enzyme activity during ice storage of fish and shellfish. Similar results were obtained in a study of succinic dehydrogenase enzyme conducted on shrimp stored in ice (Sajan George, 1979).

3.2.7 ENZYME ACTIVITY AND MUSCLE pH IN ICE STORED FISH/SHELL-FISH

Significant increase in muscle pH of mullet, pearlspot, tilapia and prawn was observed with time of ice storage. Although there was slight increase in muscle pH in milkfish, this was not found statistically significant. Most rapid increase in muscle pH was observed in P. indicus in which case pH of the muscle at the end of 16 days ice storage reached the value of 7.65. The rise in pH of iced fish can be explained by the accumulation of basic end products of putrefaction.

Similar findings were reported by Reay and Shewan (1949). As fish pass out of rigor mortis and bacterial spoilage develops, the pH of the flesh of lean fish rises from the rigor minimum to neutrality and then beyond to 7.5 to 8 or even higher as putrefaction proceeds. Nair et al. (1971) have observed that the muscle pH of the fresh water fish mrigal (initial pH 6.30) slightly increased upto 7 days of ice storage and remained more or less constant on prolonged storage to 36 days. The pH obviously had not been affected because there was no undue accumulation of basic metabolites. It was reported earlier that the pH of prawn stored in ice increased from 6.5 to 7.3 at day 12 (Shaikhmahamud and Magor, 1965).

The spoiled marine fish is usually reported to reach pH values higher than fresh water fish (Reay and Shewan, 1949), Botta et al. (1983) reported that muscle pH of ungutted offshore capeline (M. villosus) stored in ice significantly increased from 7.12 at day 0 to 7.28 at day 16. However in the case of gold lined sea bream (Rhabdosargus sarba) stored in ice, pH value

increased from 6.2 at day 0 to 6.7 on day 31. At higher temperature ($10^{o}C$) pH increased rapidly from 6.2 to 7.5 by day 14 (Curran et al., 1980). pH value of iced scampi (Nephrops norvegicus) rapidly increased from 6.32 initially to 7.90 at day 17 (Stroud et al., 1982). It was reported for southern blue whiting (M. australis) that the ultimate muscle pH value 6.85 to 6.90 increased slowly to reach 7.05 to 7.10 after 10 days storage in ice (Barassi et al., 1981).

Dark muscle was found to have higher initial pH value than white muscle. On storage in ice, pH value of white muscle decreased very slowly whereas dark muscle suffered greater decrease in pH. Dark muscle maintained a higher pH during the whole storage period in ice than the white muscle. The lower initial pH value of the white muscle was probably due to the accumulation of large amounts of lactic acid because it has been reported that in mackeral the fresh white muscle contained much higher concentration of this acid (815 mg/100 g) than the corresponding dark muscle (380 mg/100 g) (Sakaguchi et al., 1982, Sakaguchi et al., 1984, Fraser et al., 1968).

In the present study significant negative correlation was observed between LDH and 5'AMP deaminase activities in fish muscle and pH in many of the species studied. However pH is not usually accepted as a good parameter for fish quality assessment, due mainly to the scattering of results, although it is important in relation to texture changes (Love & Haq, 1970). According to the regulations of some countries, the pH values must not be above 7.5 for fish other than elasmobranchii (Lupin et al., 1980).

3.2.8 CHANGES IN ENZYME ACTIVITY AND TOTAL VOLATILE NITROGEN CONTENT

On ice storage of fish and shellfish, it was observed that TVN content of the muscle increased with storage period. Significant positive correlation was found between TVN value and days of ice storage in all the fishes studied. Similar findings are made by Varma et al. (1983) while studying the ice storage characteristics of pearlspot, mullet and tilapia. Nair et al. (1971) report that total volatile nitrogen content of the fresh water fish mrigal rise from the initial value of 7.3 mg N_2/100g muscle to 28.8 mg N_2/100g muscle on day 35 on ice storage.

Govindan (1982) reported that on ice storage of P. indicus TVN shows a small rise after 3 days of storage and increases rapidly after 10 days. Earlier studies have shown that rapid rise in TVN value occurs as a result of ice storage of marine species (Jensen et al 1979; Curran et al. 1980; Sakaguchi et al. 1984; Lupin et al. 1980; Stroud et al. 1982; Sakaguchi et al 1982; Barassi et al. 1981; Krishna Kumar et al. 1985).

Total volatile nitrogen content of chill stored fish was reportedly in good agreement with organoleptic determinations (Vyncke, 1980). Cantoni et al (1978) reported that TVN/AA-N ratio is probably the most accurate index of shrimp quality because it is based on the post-mortem production of basic volatile nitrogen by both bacteria and enzymes. It can be influenced by volatile and amino acid nitrogen following the loss of liquid from muscular tissue after death.

In the present study a consistent relationship was observed between TVN

content and activities of the fish muscle enzymes, LDH and 5'AMP deaminase. Significant positive correlation was observed between the above two factors in all the samples. However it is difficult to prescribe an uniform index of incipient spoilage because of variations among species. Significant correlation was observed between 5'AMP deaminase activity and TVN content in Pink (P. duorarum) and brown shrimp (P. azetecus) by Cheuk et al (1979).

Ice storage resulted in rapid loss of TVN and ∝amino nitrogen in P. indicus. The phenomenon of leaching out the water soluble nitrogen and free amino nitrogen from the muscle of ice stored prawns have been reported earlier (Govindan, 1982). Leaching out of soluble nitrogenous compounds from the muscle into the ice melt water is accompanied by the muscle absorbing water from melting ice. Basu and Khasim (1985) observed during a comparative ice storage study of milk fish (Chanos chanos) that the leaching effect is more significant on TVN and NPN than on ∝amino nitrogen components. However in the case of blood clam (Anadara granosa) meat both TVN and ∝amino nitrogen values showed decreasing trend owing to leaching by ice melt water (Basu & Gupta, 1984).

CHAPTER 4

STABILITY OF ENZYMES IN FISH/SHELLFISH SUBJECTED TO COLD STORAGE

4.1 RESULTS

4.1.1 EFFECT OF FREEZING AND THAWING ON ENZYME ACTIVITY IN PRESS JUICE OF FISH MUSCLES

The relation between enzyme activity in press juice of fish muscles before and after freezing and thawing is shown in Fig. 9. Freezing and subsequent thawing of fresh fish have resulted in an increase in enzyme activity in press juice of fish muscles. Freezing caused only a marginal increase in Ca^{2+} ATPase activity in press juice of muscles. Substantial rise in press juice lipoamide reductase activity was observed with most of the species subjected to freezing. With the species milkfish, 70.5% rise in lipoamide reductase activity was observed on freezing and thawing. Substantial increase was found in specific activity of LDH in press juice of muscles of samples belonging to the species pearlspot and milk fish. Although increase in specific activity was seen with other species, this was not significant.

Freezing and thawing caused an increase in 5' AMP deaminase activity in the press juice of muscles of all the six species subjected to frozen storage. With the species pearlspot 85% increase in press juice activity occured as a result of freezing and thawing. Percent increase in specific activity was 73.5 with the species mullet. Only marginal increase in 5' AMP deaminase activity was observed with the remaining species such as mrigal, milkfish, tilapia and P. indicus. Lactate dehydrogenase activity in the press juice of fish muscles also showed more or less similar influence on freezing and thawing.

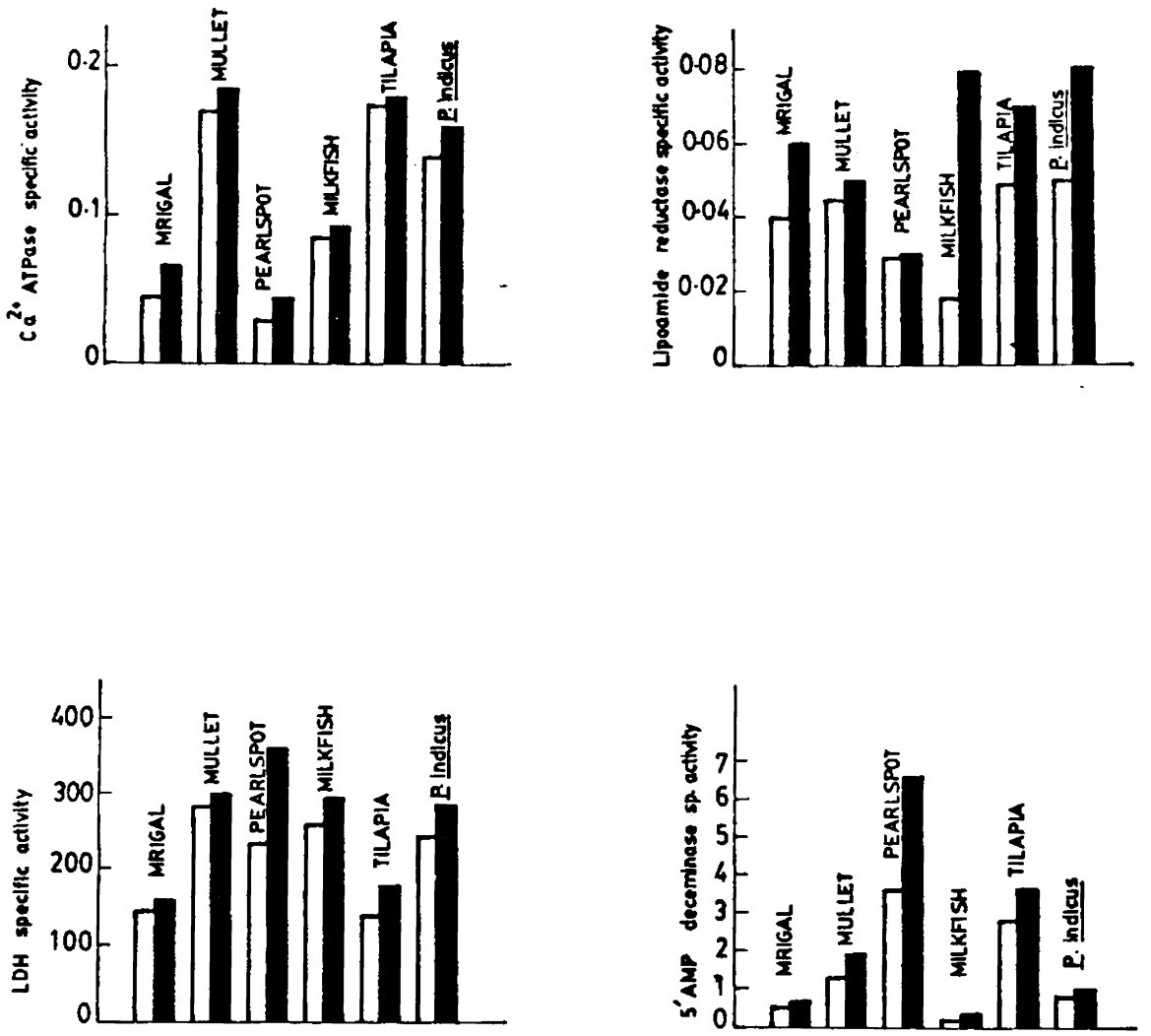

FIGURE 9. Histograms showing the effect of freezing and thawing on the activities of press juice enzymes in the six species of fish and shellfish. Figure shows specific activity of the enzymes in press juice of muscles from fresh fish (□) and frozen thawed fish (■)

4.1.2 STABILITY OF CA^{2+} ATP_{ase} IN FISH/SHELLFISH MUSCLE DURING FROZEN STORAGE

Ca^{2+} ATPase activities in fish muscle extract and press juice of muscles of different species of fish and shellfish were periodically assayed in order to ascertain the trend in change of activity. Whole fish and shellfish were frozen and stored at $-20 \pm 2^{o}C$ for a period of six months. Samples were with drawn on every 15 days interval and tested for enzyme activity. Portions of white muscles were removed from middle and dorsal part of the body. Part of them were used for the preparation of muscle extract and the remaining were used for press juice preparation. Muscle extract was prepared in 0.6 M KCl containing 0.01 M $NaHCO_3$ adjusted to pH 8.0. The extract was placed in a refrigerator for 1 hr with occasional stirring. Centrifuged at 3000 rpm for 15 minutes and supernatant was used for enzyme assay.

Effect of frozen storage on the activity of Ca^{2+} ATPase in the muscles of fish and shellfish belonging to different species was studied and the results are shown in Fig.10. Decrease in Ca^{2+} ATPase activity in muscle extract was found in all the samples subjected to frozen storage. Highly significant fall in activity was shown by the species mullet. Mullet muscle showed a specific activity of 0.125 initially. Enzyme activity was completely lost at the end of 165 days of frozen storage. Ca^{2+} ATPase activity in the muscle extract of mrigal and tilapia also suffered appreciable loss as a result of frozen storage. With certain species such as pearlspot, an initial increase in activity was caused by frozen storage.

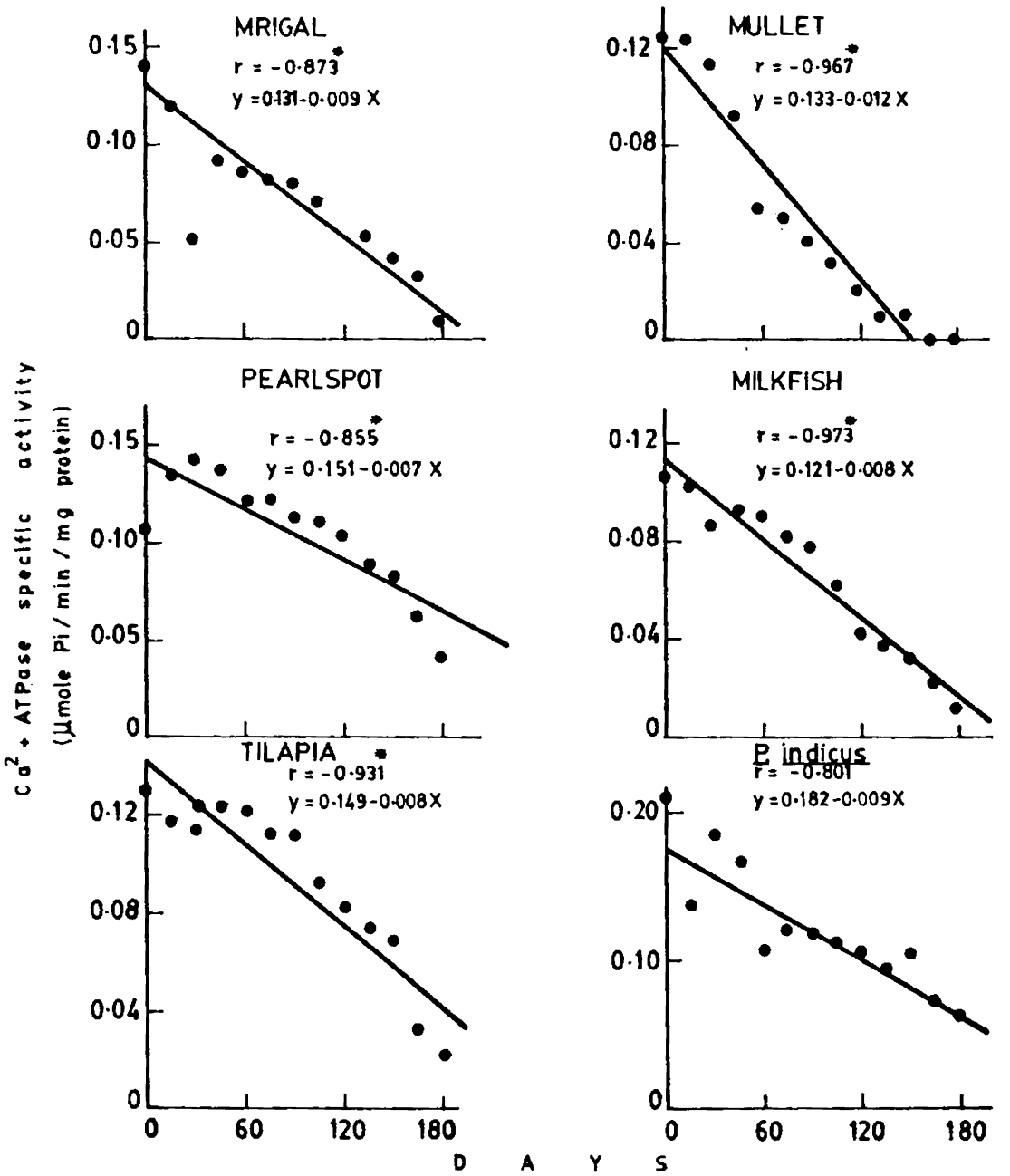

FIGURE 10. Stability of Ca^{2+} ATPase in the muscle of fish and shellfish subjected to frozen storage. Correlation coefficients and regression lines showing the relationship between muscle Ca^{2+} ATPase activity and storage period are also shown in the figure. Significance level, * $P \leq .05$ (5% level)

Ca^{2+} ATPase activity in press juice of fish muscles also suffered loss as a result of frozen storage. Considerable fall in activity was observed in mullet, milk fish, tilapia and P. indicus at the end of 180 days frozen storage. Specific activity of the enzyme was 0.170 in press juice of fresh mullet muscle. At the end of six months frozen storage specific activity had fallen to 0.021. Similarly with the species tilapia, initial activity of 0.174 has significantly decreased to 0.010 as a result of frozen storage.

Ca^{2+} ATPase activity in the species mrigal and pearlspot registered a slight increase during early days of frozen storage which on further storage decreased.

4.1.3. CORRELATION BETWEEN CA^{2+} ATPase ACTIVITY IN FISH MUSCLE AND PERIOD OF FROZEN STORAGE

The trend in change of Ca^{2+} ATPase activity with period of frozen storage was determined by estimating regression lines and determining correlation coefficients. Correlation between Ca^{2+} ATPase activity and period of frozen storage is shown in Fig. 10. Negative correlation existed between Ca^{2+} ATPase activity in fish muscle extract and storage period. Highly significant negative correlation ($P \leq 0.01$) was observed between enzyme activity and storage period with the species mullet ($r = -0.967$) and milkfish ($r = 0.973$). Although negative correlation was observed between enzyme activity and period of frozen storage in the species pearl spot and P. indicus this was not found statistically significant.

Correlation between Ca^{2+} ATPase activity in press juice of muscles and period of frozen storage has been determined in the six species of fish and shellfish. Results are shown in Fig. 11. Significant negative correlation existed between

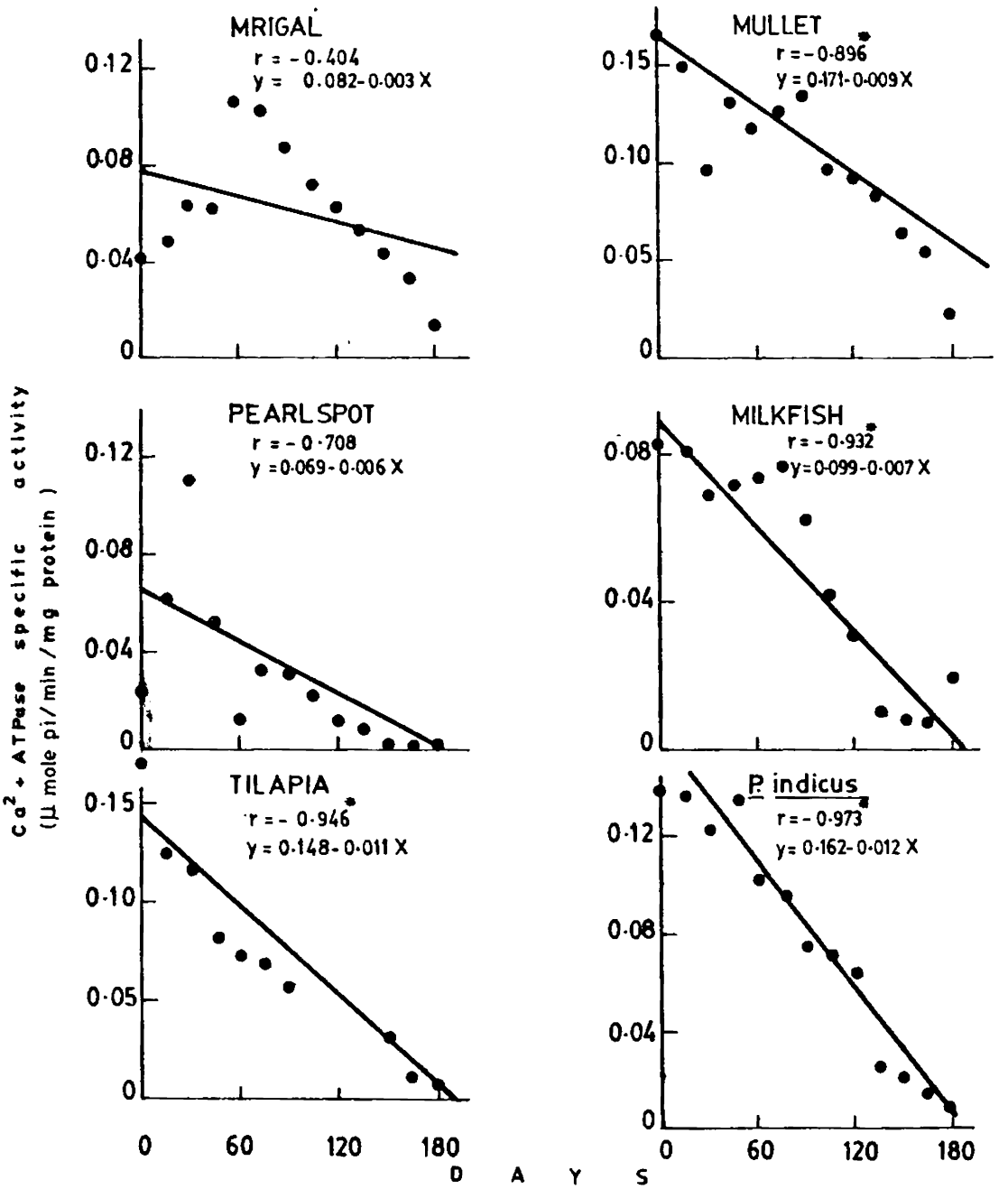

FIGURE 11. Stability of Ca^{2+} ATPase in the press juice of muscles of fish and shellfish subjected to frozen storage. Correlation coefficients and regression lines showing the relationship between press juice Ca^{2+} ATPase activity and frozen storage period are also shown in the figure. Significance level, * $P \leq 0.05$ (5% level)

Ca^{2+} ATPase activity and frozen storage period in the species mullet (r = -0.896) and milk fish (r = -0.932) while highly significant (P .01) correlation existed in tilapia (r = -0.946) and P. indicus (r = -0.973). Although negative correlation between the above two variables was observed in mrigal and pearl-spot this has not been found statistically significant.

4.1.4 CORRELATION OF CA^{2+} ATPase ACTIVITY IN FISH MUSCLE AND TESTS OF FRESHNESS

Correlations between Ca^{2+} ATPase activity in fish muscle and some of the common tests of freshness have been determined. Such an attempt was made in order to assess the reliability of enzyme assay as a test of freshness of fish in comparison with other established sensory and chemical indices. Samples were with drawn from the frozen lot at every 15 days interval and using one portion, enzyme activities were determined. On the another portion, freshness tests such as sensory score, pH, TBC, α amino nitrogen, FFA, PV & TBA number were conducted. Correlation coefficients between Ca^{2+} ATPase activity and each of these freshness parameters have been computed according to the method described in Chapter-I.

Table 33 illustrates the linearity of the relationship between Ca^{2+} ATPase activity and freshness indices in the six species of fish and shellfish studied. The shellfish (P. indicus) alone is giving uniformly correlative values. All the fish species generally show either significant or highly significant linear relations. Among the various indices tested, over all acceptability score gives excellent positive correlation with enzyme activity. Similar is the case with the indices of rancidity such as FFA and PV. One exception is in the case of mrigal. Also the TBC test is not giving significant correlation in any of the species.

TABLE 33. Correlation coefficient matrix between Ca^{2+} ATPase activity in fish muscle extract and freshness tests in the six species of fish and shellfish subjected to frozen storage. Significance level* $P \leq 0.005$ **$P \leq 0.01$

No.	Freshness Tests	Correlation Coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.741	-0.967**	-0.935**	-0.968**	-0.932**	-0.790
2.	TBC	0.501	0.281	-0.954**	-0.322	-0.736	0.604
3.	α amino N_2	0.727	0.880*	0.896*	0.901*	0.905*	-0.444
4.	TVN	-0.840*	-0.965**	-0.725	-0.899*	-0.930**	-0.765
5.	FFA	-0.853*	-0.966*	-0.830*	-0.924**	-0.851*	0.130
6.	PV	-0.814*	-0.913*	-0.828*	-0.975**	-0.917**	0.802
7.	TBA number	-0.734	-0.944**	-0.878*	-0.962**	-0.929**	-0.754
8.	Overall acceptability score	0.885*	0.966**	0.831*	0.929**	0.950**	0.780

TABLE 34. Correlation coefficient matrix between Ca^{2+} ATPase activity in press juice of fish muscles and freshness tests in the six species of fish and shellfish frozen stored. Significance level * $P \leq 0.05$ ** $P \leq 0.01$

No.	Freshness Tests	Correlation Coefficients (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.348	-0.872*	-0.718	-0.895*	-0.858*	-0.548
2.	TBC	-0.093	0.080	-0.670	-0.187	-0.458	0.207
3.	∝ amino nitrogen	0.725	0.910*	0.661	0.872*	0.593	0.353
4.	TVN	-0.477	-0.858*	-0.514	-0.839*	-0.908*	-0.974**
5.	FFA	-0.536	-0.878*	-0.687	-0.860*	-0.947**	-0.958**
6.	PV	-0.316	-0.883*	-0.042	-0.947**	-0.839*	-0.978**
7.	TBA Number	-0.076	-0.803*	-0.775	-0.962**	-0.242	-0.929**
8.	Overall accept ability score	0.237	0.836*	0.593	0.923**	0.136	0.433

The relationship between Ca^{2+} ATPase activity in press juice of muscles and freshness indices in six species of fish and shellfish was studied using Karl Pearson's correlation coefficients. Results are shown in Table 36. In the case of mullet and milk fish significant correlations were observed except with TBC test. In the case of tilapia only pH, TVN, FFA and PV are showing significant results. Unlike in the case of enzyme activity in muscle extract, in the press juice of P. indicus highly significant correlation has been observed for TVN, FFA, PV and TBA number tests. However, in the other tests the correlation coefficients are not significant. The overall acceptability score test is showing significant results only for mullet and milk fish. In the case of mrigal and milk fish none of the correlation values was significant.

4.1.5 EFFECT OF FROZEN STORAGE ON LIPOAMIDE REDUCTASE ACTIVITY IN SIX SPECIES OF FISH AND SHELLFISH.

Lipoamide reductase activity was determined both in fish muscle extract and press juice of muscles, of different species of fish and shellfish during the period of frozen storage. Results are shown in Fig. 12 and Fig. 13. Fish muscle showed low lipoamide reductase activity. Considerable loss in lipoamide reductase activity was observed with all the six species of fish and shellfish subjected to frozen storage. Among the different species studied, greatest fall in activity was observed in pearlspot. In this case residual activity at the end of 180 days' frozen storage was 5.12%. This was followed by mrigal (residual activity 5%). Lipoamide reductase activity in tilapia was found least affected by frozen storage.

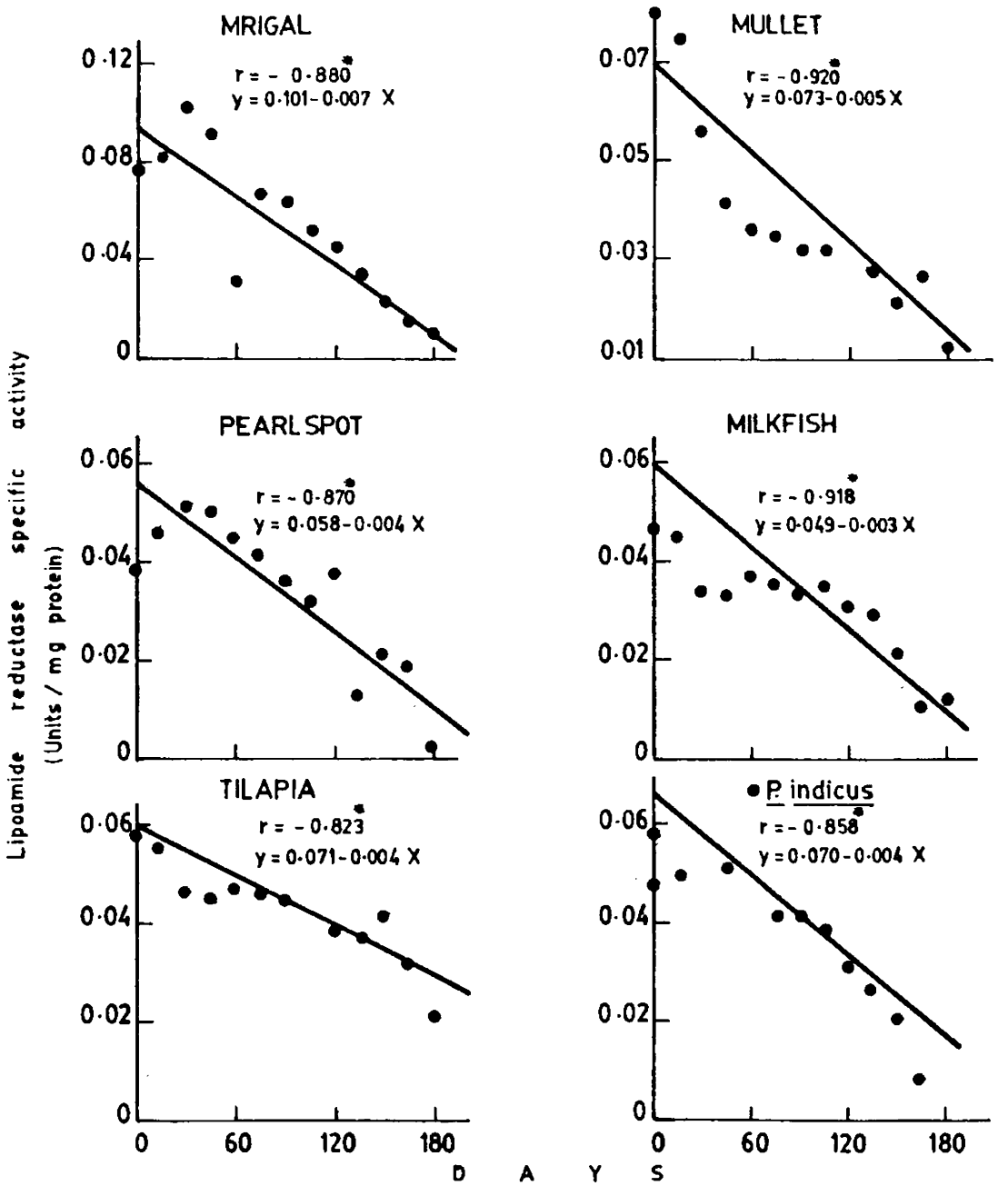

FIGURE 12. Stability of lipoamide reductase in the muscle of fish and shellfish subjected to frozen storage. Correlation coefficients and regression lines showing the relationship between lipoamide reductase activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

Steady decline in lipoamide reductase activity on frozen storage was seen only in mullet, milk fish and tilapia. In other species the enzyme activity initially increased and this was followed by steady loss in activity. Disruption of the tissue by a freeze thaw cycle might solubilise the enzyme and this can be the reason for recording higher enzyme activity initially.

Press juice enzyme has undergone similar pattern of change during frozen storage. Fig. 13 shows that considerable loss in enzyme activity has resulted in most of the species frozen stored. Total disappearance of enzyme activity was seen in mullet and milk fish at the end of 6 months frozen storage. In samples of the species mrigal, residual activity at the end of 180 days frozen storage was 5%. Enzyme activity in pearl spot and tilapia was least affected as a result of frozen storage.

Only in mullet a progressive decrease in lipoamide reductase activity in press juice was observed with storage period. In the rest of the species enzyme activity initially rose and on further storage decreased.

4.16 CORRELATION BETWEEN LIPOAMIDE REDUCTASE ACTIVITY AND STORAGE PERIOD

Regression lines of lipoamide reductase activity on storage period in different species of fish and shellfish are shown in Fig.12. Highly significant correlation of lipoamide reductase activity with storage period has been obtained in all the six species of fish and shellfish studied. Changes in muscle lipoamide reductase activity in mullet and milkfish was inversly proportional to increase in storage period. Significant negative correlation ($P \leq 0.05$) was shown by lipoamide reductase activity in muscle extract and frozen storage

TABLE 35. Correlation coefficient matrix between lipoamide reductase activity in muscle extract and freshness indices in the six species of fish and shellfish frozen stored. Significance level * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation Coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.775	-0.906*	-0.932**	-0.580	-0.787	-0.838*
2.	TBC	0.335	0.252	-0.912*	-0.349	-0.547	0.726
3.	∝ amino nitrogen	0.286	0.786	0.897*	0.476	0.770	0.588
4.	TVN	-0.871*	-0.937**	-0.427	-0.731	-0.831*	-0.848*
5.	FFA	-0.887*	-0.927**	-0.829*	-0.875*	-0.892*	-0.831*
6.	PV	-0.836*	-0.889*	-0.844*	-0.299	-0.806	-0.734
7.	TBA	-0.492	-0.938**	-0.788	-0.768	-0.851*	-0.821*
8.	Overall acceptability score	-0.816*	0.915*	0.865*	0.909*	0.864*	0.859*

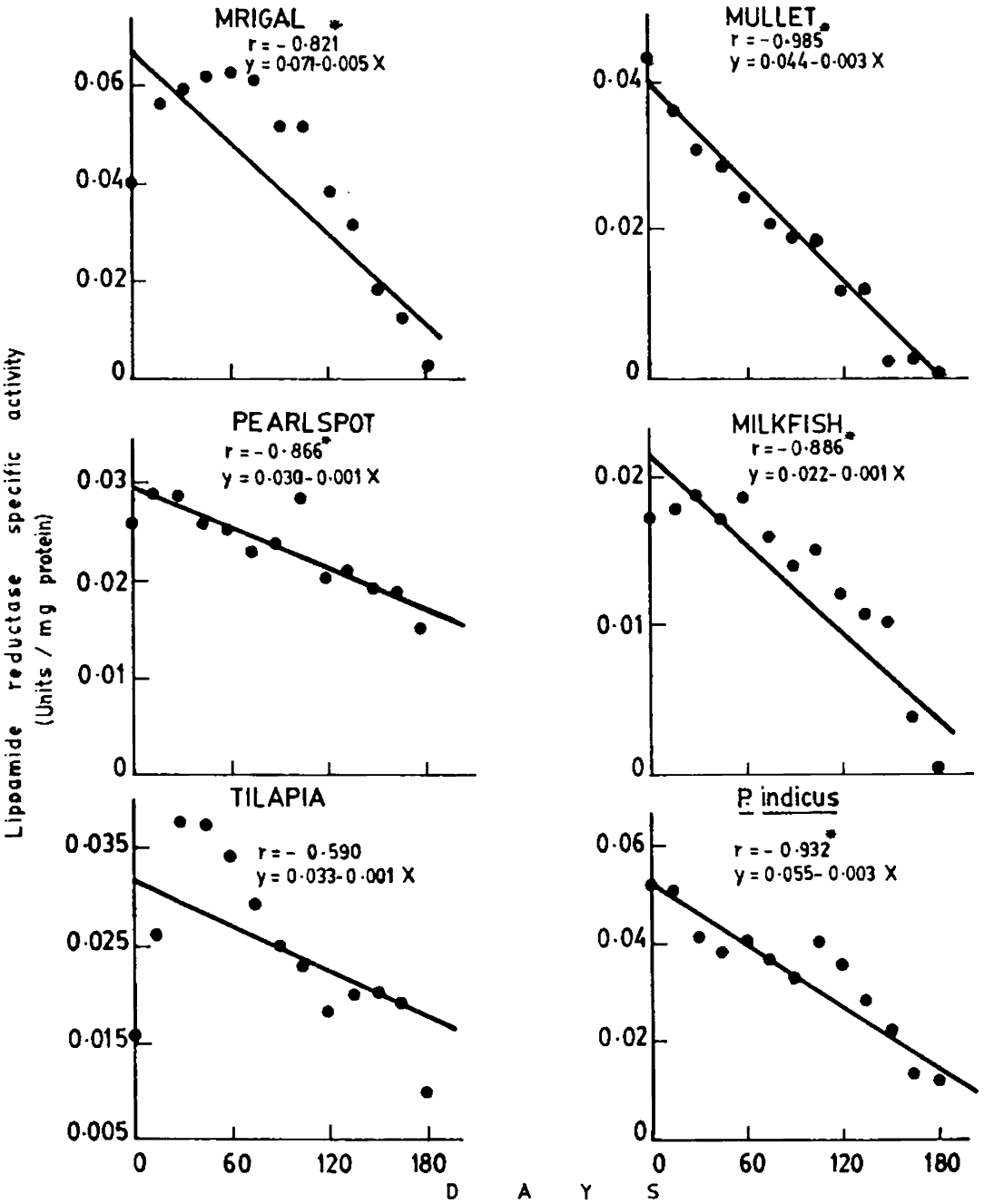

FIGURE 13. Stability of lipoamide reductase in the press juice of muscles of fish and shellfish subjected to frozen storage. Correlation coefficients and regression lines showing the relationship between press juice lipoamide reductase activity and storage period are also shown in the figure. Significance level, * $P \leq 0.05$ (5% level)

period in fishes belonging to the species mrigal, pearl spot, tilapia and P. indicus.

Negative correlation was seen between changes in lipoamide reductase activity in press juice and storage period. Simple correlation between the two variables has been worked out and was found that correlation coefficient was significant at 5% level ($P \leq 0.05$) in samples of the species mrigal, mullet, pearlspot, milkfish and P. indicus. Among these species, highly significant correlations ($P \leq 0.01$) were seen with mullet and P. indicus. However, no significant negative correlation could be observed in tilapia.

4.1.7 CORRELATION BETWEEN LIPOAMIDE REDUCTASE ACTIVITY AND FRESHNESS TESTS

The correlation coefficients between lipoamide reductase activity in fish muscle extract and freshness tests for the six species of fish/shellfish were worked out to establish the relationship between them. The results are shown in Table 35. These correlations were not uniformly significant within any one of the species under different freshness tests. But in most of the cases, the values were negative. Of the different freshness tests carried out FFA and overall acceptability score alone gave significant correlation values with muscle lipoamide reductase activity.

In order to study the nature of relationship between lipoamide reductase activity in press juice of fish muscles and freshness tests, Karl Pearson's coefficients of linear correlations were worked out. Results are shown in Table 36. In majority of the cases these correlation values were not significant though negative relations were dominating. The overall acceptability test gave significant results uniformly except in the case of tilapia.

TABLE 36. Correlation coefficient matrix between lipoamide reductase activity in press juice of fish muscles and freshness test values in the six species of fish and shellfish fish frozen stored. Significance level, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation Coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.850*	-0.977**	-0.477	-0.925**	-0.522	-0.935**
2.	TBC	-0.029	0.396	0.476	-0.099	-0.281	0.099
3.	∝ amino nitrogen	-0.120	0.445	0.831*	0.802	0.755	0.416
4.	TVN	-0.904*	-0.913*	-0.848*	-0.892*	-0.563	-0.937**
5.	FFA	-0.330	-0.967**	-0.834*	-0.848*	-0.538	-0.965**
6.	PV	-0.867*	-0.983**	-0.831*	-0.879*	-0.670	-0.796
7.	TBA	-0.336	-0.900*	-0.751	-0.751	-0.653	-0.771
8.	Overall acceptability score	0.876*	0.975**	0.836*	0.840*	0.633	0.967

4.1.8 STABILITY OF LACTATE DEHYDROGENASE IN FISH/SHELLFISH MUSCLE DURING FROZEN STORAGE

Considerable loss in LDH activity was seen in all the species of fish and shellfish subjected to frozen storage. Fig. 14 shows values of lactate dehydrogenase activity plotted against period of frozen storage. Frozen storage caused significant loss in LDH activity in most species studied. Loss in LDH activity in P. indicus muscle was 90% of initial activity at the end of 15 days storage. LDH activity in prawn muscle was completely lost at the end of 135 days frozen storage. LDH activity in tilapia also suffered considerable loss on prolonged cold storage. Steady decrease in LDH activity was seen in mrigal, mullet, pearlspot and milk fish when subjected to frozen storage.

LDH activity in press juice of muscles of most of the species studied suffered considerable loss when subjected to frozen storage (Fig.15). In many of the species, press juice enzyme activity registered an increase during the initial period of frozen storage followed by gradual decrease in activity.

4.1.9 CORRELATION BETWEEN LDH ACTIVITY AND FROZEN STORAGE PERIOD

Regression lines of LDH activity in different species of fish/shellfish on storage period are shown in Fig.14. Very high correlation ($P \leq 0.01$) of LDH activity with storage period was observed with mrigal, mullet, pearlspot, milkfish and tilapia. LDH activity significantly decreased with period of frozen storage. Although negative correlation was seen between LDH activity and frozen storage period with the species P. indicus this was not found statistically significant.

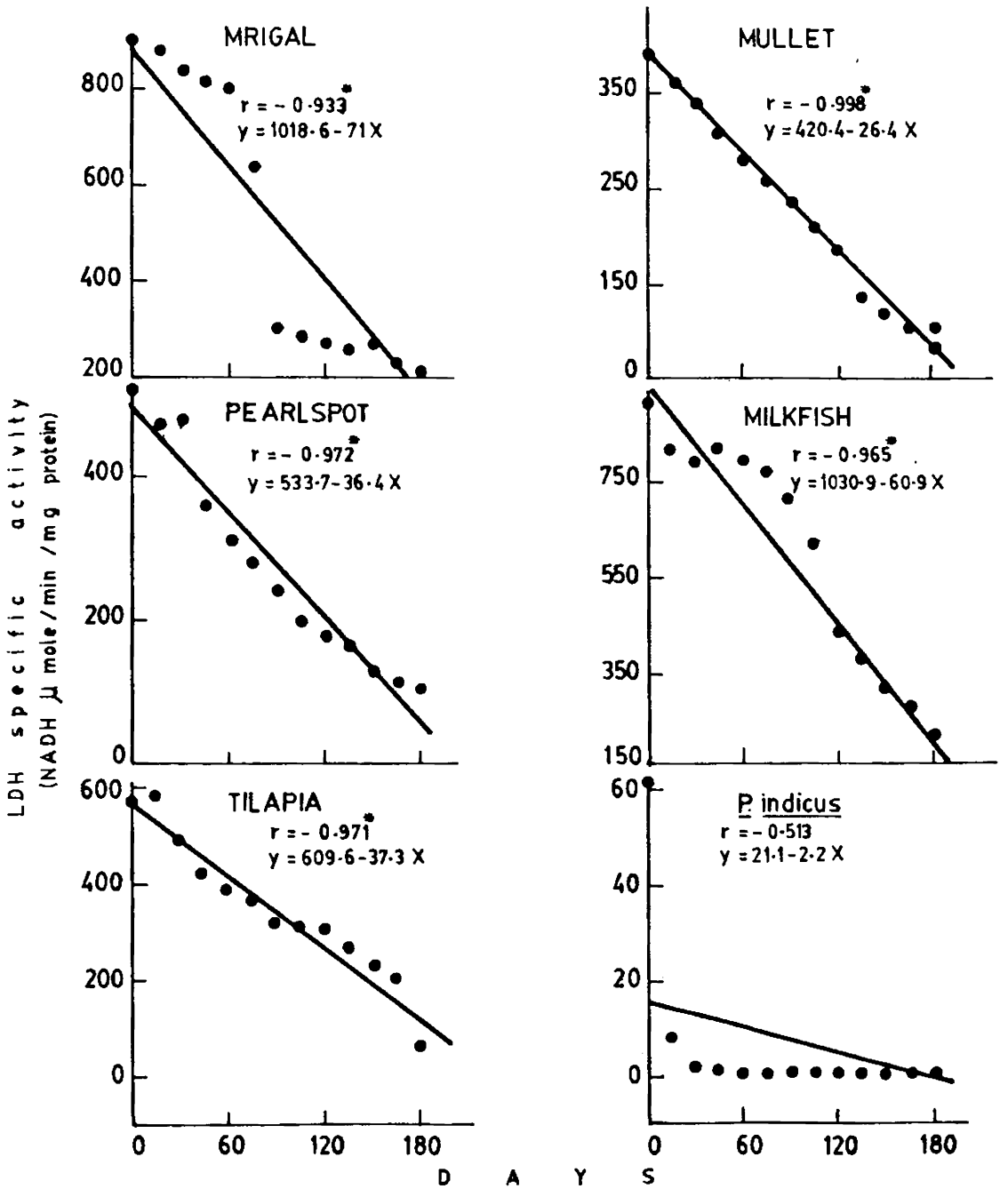

FIGURE 14. Stability of lactate dehydrogenase in the muscle of fish and shellfish subjected to frozen storage. Correlation coefficients (r) and regression lines showing the relationship between lactate dehydrogenase activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

Significant negative correlation between LDH activity in press juice of muscles and frozen storage period was seen in mullet, pearlspot, milkfish and P. indicus. Although negative relations were observed with the species mrigal and tilapia, correlation values were not significant.

4.1.10 CORRELATION BETWEEN LDH ACTIVITY AND FRESHNESS TESTS

The correlation coefficients between LDH activity in fish muscle extract and various freshness test values were worked out. The results are presented in the Table 37. Significant correlation values were seen under various tests except TBC test for most of species of fish. Noticeably for mrigal alone the correlation values are not significant under pH, TBA number and overall acceptability score in addition to TBC test. But for the shell fish, P. indicus no correlation value was significant. In the case of tilapia the correlation value under α-amino nitrogen test was not significant.

The relationship between LDH activity in press juice of fish muscle and freshness test values on different species of fish and shell fish subjected to frozen storage is shown in Table 38. On examination of the Table, it can be noted that none of the correlation values are significant for tilapia. In the case of prawn, only the value under pH test gave a significant result. With the other species invariably significant results are observable. Again the TBC test was not yielding significant results in most of the cases (only in pearlspot).

4.1.11 STABILITY OF 5' AMP DEAMINASE ACTIVITY IN FISH/SHELLFISH MUSCLE DURING FROZEN STORAGE

Fig.16 shows that 5' AMP deaminase activity in fish muscle extract significantly decreases on prolonged cold storage. Among the different species

TABLE 37. Correlation coefficient matrix between LDH activity in fish muscle extract and freshness test values in the six species of fish and shellfish subjected to frozen storage. Significance level, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.740	-0.985**	-0.896**	-0.944**	-0.901*	-0.450
2.	TBC	0.136	0.146	-0.736	-0.320	-0.659	-0.103
3.	αamino nitrogen	0.839*	0.948**	0.818*	0.893*	0.723	-0.600
4.	TVN	-0.902*	-0.863*	-0.916**	-0.882*	-0.951**	-0.421
5.	FFA	-0.902*	-0.986**	-0.971**	-0.914*	-0.842*	-0.415
6.	PV	-0.975**	-0.966**	-0.971**	-0.972**	-0.826**	-0.469
7.	TBA number	-0.676	-0.977**	-0.937**	-0.979**	-0.933**	-0.384
8.	Overall acceptability score	0.799	0.979**	0.898*	0.929**	0.884*	0.458

TABLE 38. Correlation coefficient matrix between LDH activity in press juice of fish muscles and freshness test values in the six species of fish and shellfish frozen stores. Significance level, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.779	-0.913*	-0.851*	-0.909*	-0.419	-0.819*
2.	TBC	-0.207	0.294	-0.820*	-0.328	-0.522	0.257
3.	α amino nitrogen	0.978**	0.957**	0.843*	0.894*	0.985**	-0.673
4.	TVN	-0.870*	-0.886*	-0.791	-0.823*	-0.448	-0.792
5.	FFA	-0.847*	-0.923**	-0.866*	-0.851*	-0.282	-0.707
6.	PV	-0.910*	-0.958**	-0.868*	-0.925**	-0.350	-0.808
7.	TBA number	-0.418	-0.962**	-0.904*	-0.949**	-0.467	-0.721
8.	Overall acceptability score	0.857*	0.927**	0.763	0.875*	0.614	0.634

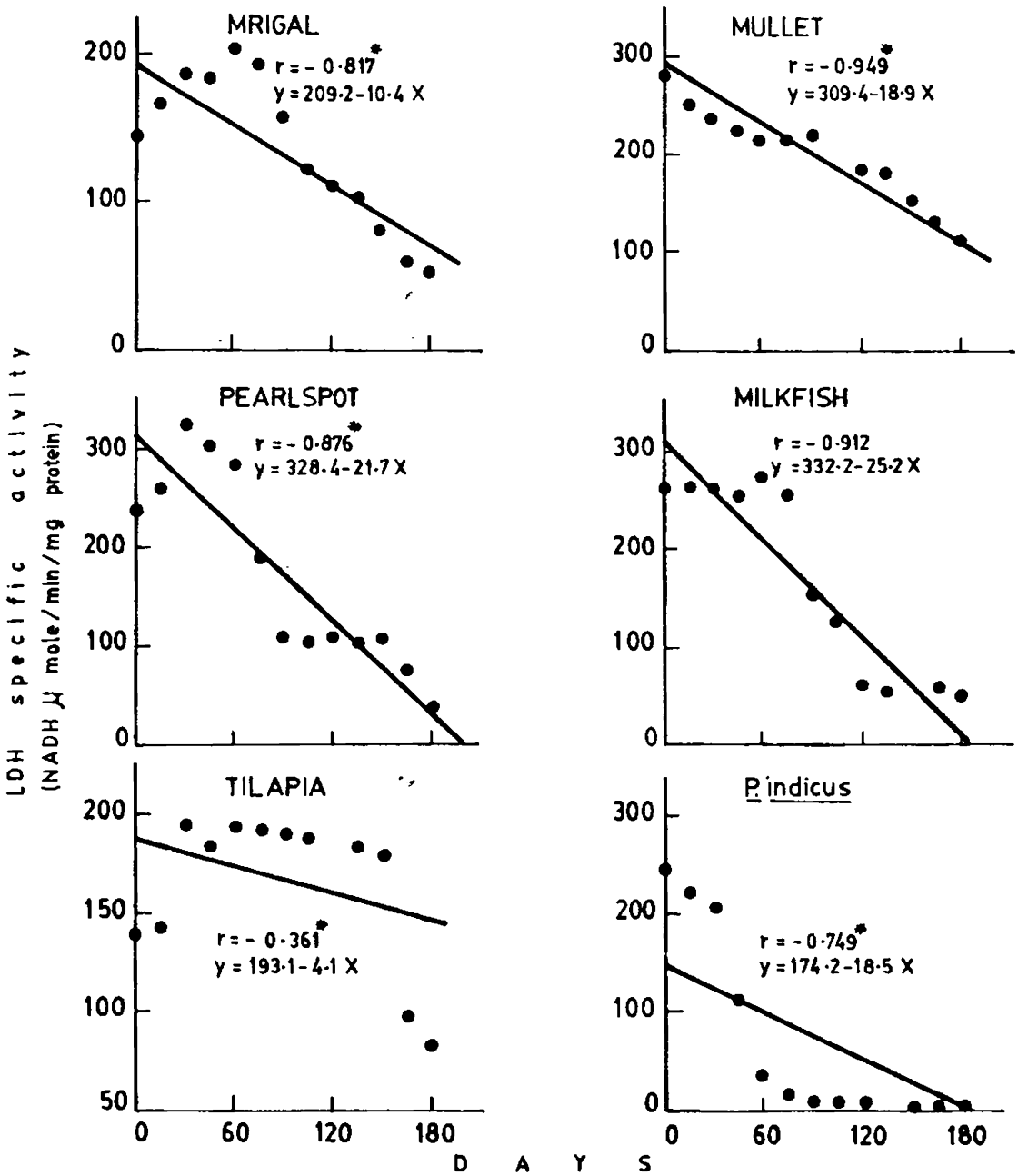

FIGURE 15. Stability of lactate dehydrogenase in the press juice of muscles of fish and shellfish subjected to frozen storage. Correlation coefficients and regression lines showing the relationship between LDH activity and storage period are also given in the figure. Significance level, * $P \leq 0.05$ (5% level)

studied, it was observed that enzyme specific activity in mrigal, milk fish and P. indicus were greatly affected by frozen storage. With the species milk fish 78.9% loss in enzyme activity occured at the end of 15 days frozen storage and at the end of 90 days, 97.4% activity was lost. Complete loss in enzyme activity occured in milk fish and prawn at the end of 105 days frozen storage. Specific activity of 5' AMP deaminase in fresh mrigal was 1.79. Enzyme activity in mrigal completely disappeared at the end of 135 days frozen storage. The species mullet and tilapia showed considerable resistance towards frozen storage. Loss of specific activity in mullet and tilapia at the end of 180 days frozen storage was 61.2% and 50.1% respectively.

Frozen storage studies indicate that progressive fall in enzyme activity takes place on prolonged cold storage. In the species milk fish and P. indicus fall in enzyme activity was so rapid that at the end of 105 days frozen storage, 5' AMP deaminase activity in fish muscle was completely lost.

The results shown in Fig. 17 indicate that the effect of frozen storage on press juice 5' AMP deaminase activity is similar to that of the enzyme in muscle extract. It can be seen from the figure that frozen storage caused considerable loss in enzyme activity with the species mrigal, milk fish, tilapia and P. indicus. 5' AMP deaminase activity was completely lost in press juice of muscles of the species milk fish, mrigal and P. indicus at the end of 19 weeks storage period. Enzyme activity in mullet and pearl spot registered comparatively greater resistance towards frozen storage. Only in mrigal and milk fish, negative correlation obtained was not found statistically significant.

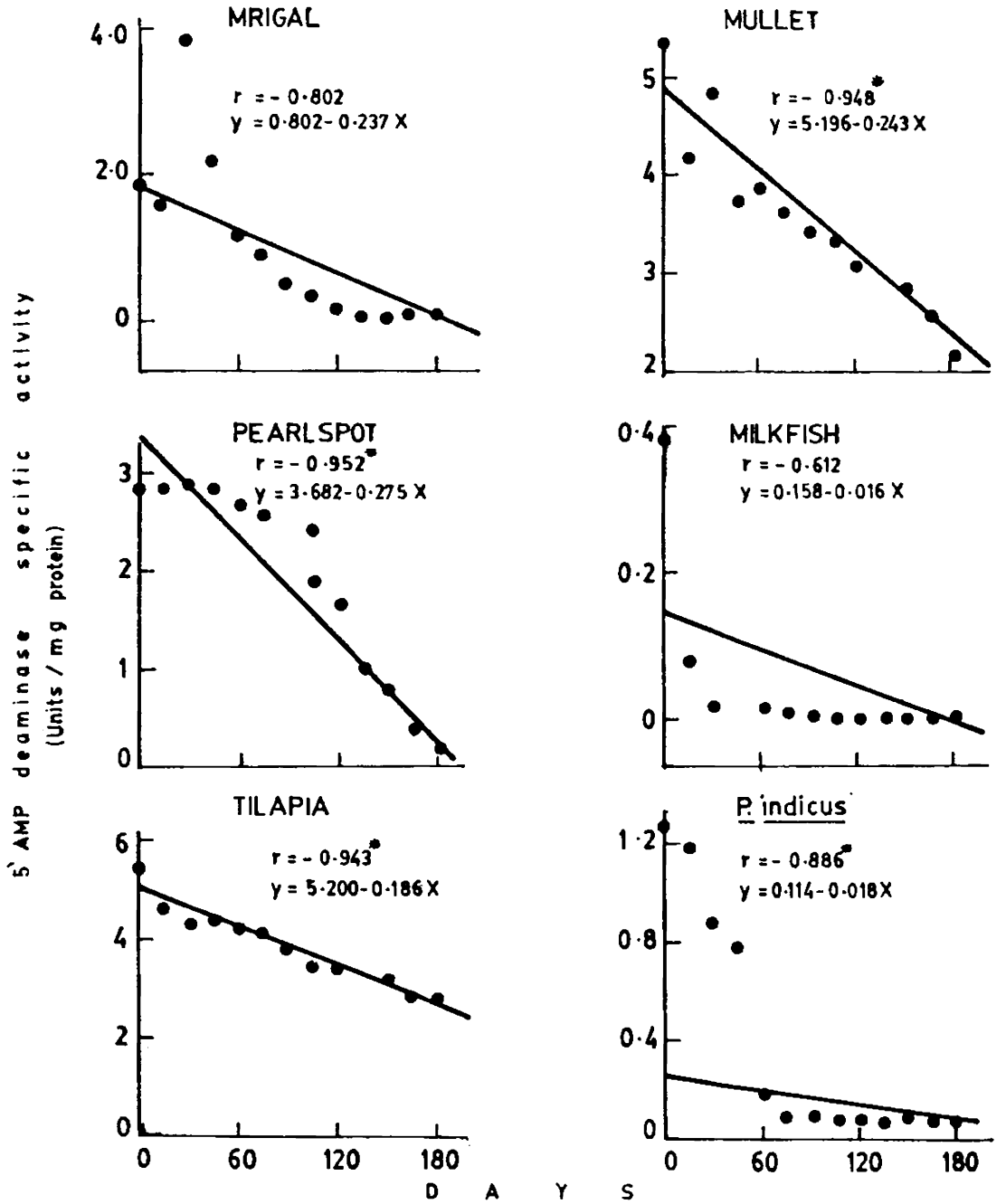

FIGURE 16. Stability of 5' AMP deaminase in the muscle of fish and shellfish subjected to frozen storage. Correlation coefficients (r) and regression lines showing the relationship between enzyme activity and frozen storage period are also shown in the figure. Significance level, * $P \leq 0.05$ (5% level)

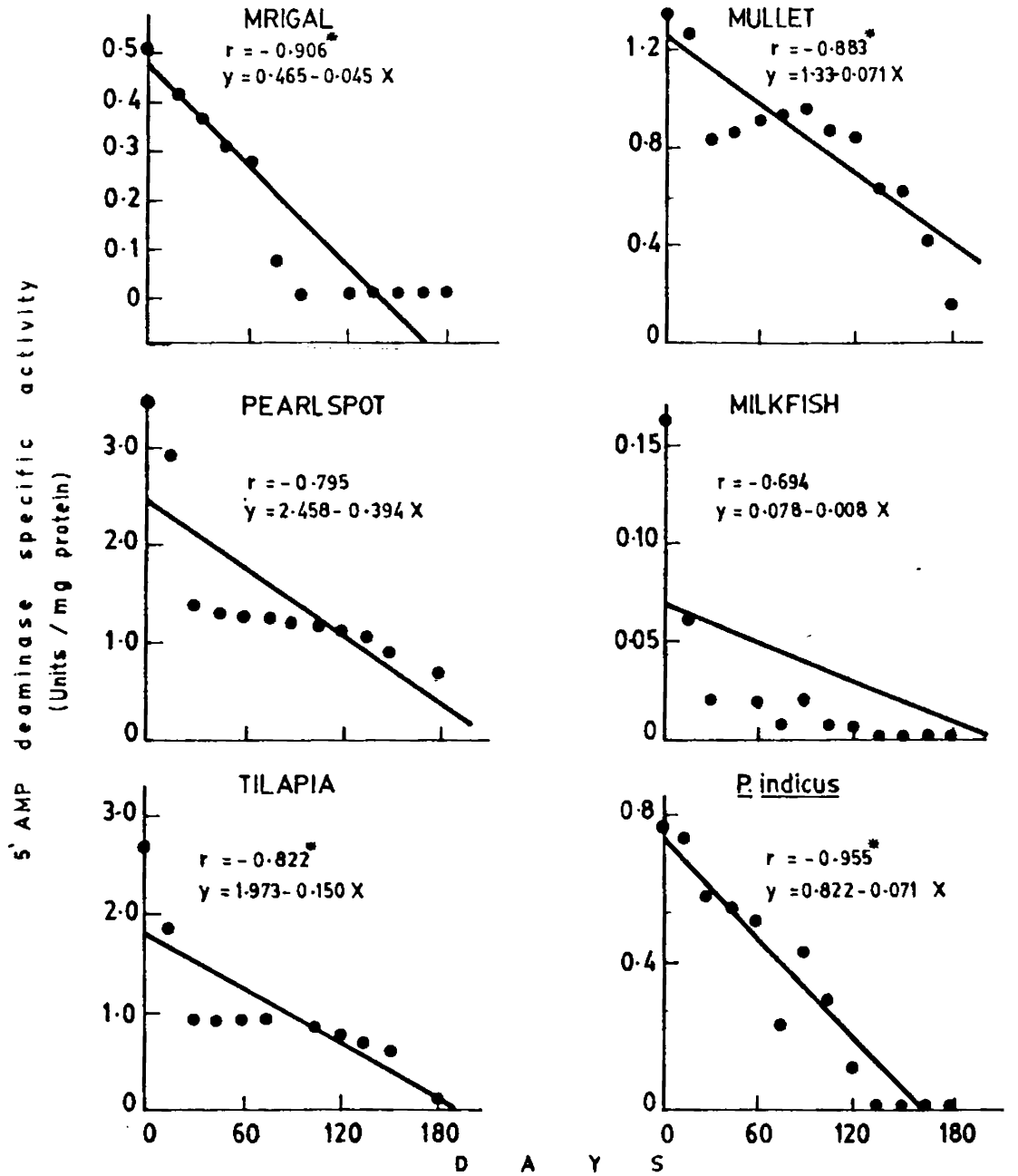

FIGURE 17. Stability of 5' AMP deaminase in the press juice of muscles of fish and shellfish subjected to frozen storage. Correlation coefficients (r) and regression lines showing the relationship between 5' AMP deaminase activity and storage period are also given in the figure. Significance level,
* $P \leq 0.05$ (5% level)

The trend in change of press juice AMP deaminase activity in fish muscle with storage period was determined by estimating regression lines and determining correlation coefficients. The results are shown in Fig.17. Highly significant negative correlation ($P \leq 0.01$) was seen with the species P. indicus where as negative correlation significant at 5% level ($P \leq 0.05$) was seen with mrigal, mullet and tilapia. Although negative relation has been observed with the species pearl spot and milk fish, correlation values were not significant.

4.1.12 CORRELATION BETWEEN 5' AMP DEAMINASE SPECIFIC ACTIVITY AND FRESHNESS TESTS

Relationship between 5' AMP deaminase specific activity in fish muscle and freshness test values on samples subjected to prolonged cold storage is shown in Table 39. In two species viz. mullet and pearlspot alone the correlation coefficients were significant. Even in these cases under TBC test, the values were not significant. In the case of mullet, α amino nitrogen test also gave results which were not significant. In the case of other species no uniformity regarding the test results was observable.

The correlation coefficients between 5' AMP deaminase activity in press juice of fish muscles and freshness test values on fish and shellfish subjected to frozen storage were determined and the results are shown in Table 40. Correlation values were not significant for pearlspot, milk fish and tilapia, under various tests. But in the case of the other three species, viz. mrigal, mullet and P. indicus some of the values were significant. Also under TBC test none of the values was significant. Under α amino nitrogen test also the correlation coefficients were not significant for

TABLE 39. Correlation coefficient matrix between 5' AMP deaminase activity in fish muscle extract and freshness test values in the six species of fish and shellfish frozen stored. Significance level, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.641	-0.916**	-0.985**	-0.042	-0.870*	-0.770
2.	TBC	-0.022	0.292	-0.043	-0.331	-0.499	0.128
3.	α amino nitrogen	0.720	0.330	0.952**	0.561	0.158	-0.839*
4.	TVN	-0.797	-0.927**	-0.858*	-0.450	0.116	-0.410
5.	FFA	-0.767	-0.934**	-0.944**	-0.612	-0.087	-0.705
6.	PV	-0.809	-0.877*	-0.951**	-0.617	-0.095	-0.919**
7.	TBA number	-0.478	-0.861*	-0.872*	-0.856*	-0.884*	-0.588
8.	Overall acceptability score	0.729	0.952**	0.961**	0.597	0.872*	0.667

TABLE 40. Correlation coefficient matrix between 5' AMP deaminase activity in press juice of muscles and freshness test values in the six species of fish and shellfish subjected to frozen storage. Significance level; * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Freshness Tests	Correlation coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	-0.679	-0.847*	-0.677	-0.554	-0.690	-0.925**
2.	TBC	0.262	0.141	-0.287	0.232	-0.439	0.284
3.	∝amino nitrogen	0.690	0.860*	0.535	0.643	0.496	-0.581
4.	TVN	-0.860*	-0.845*	-0.768	-0.524	-0.784	-0.934**
5.	FFA	-0.824*	-0.867*	-0.788	-0.460	-0.781	-0.858*
6.	PV	-0.863*	-0.863*	-0.159	-0.700	-0.689	-0.945**
7.	TBA number	-0.768	-0.775	-0.668	-0.698	-0.679	-0.905*
8.	Overall acceptability score	0.726	0.825*	0.770	0.681	0.688	0.890*

mrigal and P. indicus. In the case of mrigal, the same was not significant under TBA and overall acceptability score.

4.1.13 FRESHNESS TESTS ON FISH AND SHELLFISH SUBJECTED TO COLD STORAGE

Various tests of freshness such as pH, TBC, α-amino nitrogen content, TVN, FFA, PV, TBA Number and overall acceptability score were carried out on samples of fish and shellfish drawn at regular intervals from the lot subjected to frozen storage. The result of pH determinations are shown in Table 41. It can be seen from the Table that frozen storage caused increase in muscle pH uniformly for all the six species studied. Highest rise in muscle pH was seen with the species tilapia. With the rest of the species, steady increase in muscle pH was observed.

Correlation coefficients were computed in order to determine whether a linear relationship existed between changes in muscle pH and period of frozen storage. Results are shown in Table 42. Highly significant positive correlation was seen between the two variables. It shows that increase in muscle pH is directly proportional to period of frozen storage.

Results of α-amino nitrogen content determinations in fish and shellfish are shown in Table 43. Highest α-amino nitrogen content was seen in the species, P. indicus. This value was 240.1 mg N/100 g muscle. Trend in increase or decrease in α-amino nitrogen content with storage period was determined and the results are shown in Table 42. Significant negative correlation was seen with the species mrigal, mullet, pearlspot and milk fish. In the species P. indicus a slight increase in α-amino nitrogen content was observed although this was not found statistically significant.

TABLE 41. Effect of frozen storage on muscle pH in the six species of fish and shellfish

Frozen Storage days	pH					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	6.29	6.15	6.07	5.68	6.07	6.83
15	6.33	6.21	6.11	5.70	6.09	6.82
30	6.31	6.32	6.08	5.69	6.13	6.89
45	6.29	6.30	6.12	5.72	6.12	6.90
60	6.32	6.45	6.20	5.78	6.21	6.98
75	6.38	6.53	6.21	5.82	6.38	7.01
90	6.41	6.58	6.25	5.81	6.41	7.12
105	6.39	6.56	6.31	5.92	6.58	7.08
120	6.48	6.71	6.41	6.08	6.71	7.15
135	6.31	6.81	6.48	6.15	6.92	7.21
150	6.59	6.88	6.50	6.22	7.11	7.23
165	6.63	6.82	6.70	6.48	7.07	7.28
180	6.62	6.91	6.72	6.61	7.08	7.18

TABLE 42. Correlation between storage period and freshness test values in various species of fish and shellfish. Significance level * $P \leq 0.05$ ** $P \leq 0.01$.

Sl. No.	Freshness Tests	Correlation Coefficient (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	pH	0.839*	0.985**	0.964**	0.943**	0.971**	0.988**
2.	∝amino nitrogen	-0.880*	-0.954**	-0.893*	-0.957**	-0.771	-0.596
3.	TVN	0.980**	0.963**	0.918**	0.931**	0.956**	0.977**
4.	FFA	0.956**	0.987**	0.991**	0.970**	0.971**	0.927**
5.	PV	0.961**	0.977**	0.987**	0.987**	0.968**	0.948**
6.	TBA number	0.707	0.977**	0.971**	0.945**	0.974**	0.934**

TABLE 43. Effect of frozen storage on α amino nitrogen content in the muscles of various fish and shellfish

Frozen Storage days	α amino nitrogen, mg N_2/100 g muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P.indicus
0	42.2	44.1	47.1	108.7	33.9	240.1
15	45.3	45.1	48.3	106.3	35.8	242.4
30	46.8	43.3	49.1	109.5	36.5	252.5
45	47.5	42.5	49.2	108.3	36.9	257.3
60	48.3	41.3	49.6	94.6	37.3	259.3
75	46.2	40.8	47.5	90.8	37.8	261.4
90	41.3	40.5	46.8	86.9	37.9	274.2
105	40.3	30.8	38.5	85.4	35.8	272.0
120	36.5	29.4	36.2	81.5	31.5	268.1
135	33.5	28.5	32.3	80.6	32.4	271.9
150	32.1	27.1	29.1	80.3	28.1	264.1
165	29.2	25.1	35.4	76.5	25.8	261.0
180	28.2	18.3	23.5	75.8	23.6	254.0

Total volatile nitrogen content which is an index of spoilage of fish and shellfish was determined on samples drawn at regular intervals from the frozen lot. Results are shown in Table 44.

TVN values showed increasing trend during frozen storage in all the species studied. Highest increase in TVN value was shown by the species pearlspot, in which case increase in TVN value was 140.8% at the end of 180 days frozen storage. This was followed by tilapia. Least increase in TVN value was shown by the species P. indicus.

Correlation coefficients were computed to determine the linearity of the above relationship. Results are shown in Table 42. Highly significant positive correlation between TVN content and storage period was seen in all the species studied ($P \leq 0.01$). It shows that as the storage period increases TVN content in the muscle also increase.

Table 45 shows the effect of period of frozen storage on free fatty acid content of muscle lipid. Frozen storage has resulted in a steady increase in FFA content of fish muscle. Greatest increase in FFA content was found in pearlspot followed by mrigal and tilapia. Karl Pearson's coefficients of linear correlations were worked out on the data obtained and the results are shown in Table 42. Highly significant positive correlation was seen between FFA content and storage period with all the species studied.

Peroxide values were determined at regular intervals in all the samples subjected to frozen storage and the result shown in Table 46. Steady increase in peroxide value was seen with all the species subjected to frozen storage. Greatest increase in peroxide value was found with the species pearlspot.

TABLE 44. Effect of frozen storage on Total Volatile Nitrogen content in the muscle of various fish and shellfish

Frozen Storage, days	Total volatile Nitrogen, mg N_2/100 g muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P.indicus
0	4.65	3.14	1.91	7.85	2.70	12.69
15	4.70	3.52	1.98	7.41	2.92	12.82
30	4.72	3.81	2.01	7.81	3.25	12.71
45	4.82	3.72	2.92	7.72	3.12	13.21
60	4.89	4.02	3.03	8.51	3.62	13.92
75	5.32	4.31	3.15	8.92	3.72	14.01
90	5.21	4.21	3.21	8.98	3.81	14.16
105	5.52	4.42	3.18	9.11	3.91	14.21
120	5.61	4.51	3.83	9.20	3.92	14.52
135	5.71	4.58	3.72	9.20	3.89	15.40
150	5.92	4.62	3.12	9.81	4.83	16.02
165	6.30	4.72	4.59	9.62	4.92	16.21
180	6.31	4.81	4.60	11.21	5.31	16.32

TABLE 45. Effect of frozen storage on free fatty acid content in the muscles of various fish and shellfish.

Frozen Storage, days	Free fatty acid, as % oleic acid					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.71	0.98	0.63	0.93	0.84	0.58
15	0.92	0.96	1.08	1.12	0.92	0.61
30	1.00	1.21	1.12	1.24	1.21	0.83
45	1.10	1.48	1.41	1.26	1.62	0.99
60	1.15	1.62	1.61	1.85	1.85	1.02
75	1.20	1.72	1.70	1.95	2.51	1.09
90	1.62	1.85	2.01	2.04	2.42	1.12
105	1.82	2.08	2.37	2.12	2.58	1.18
120	2.91	2.21	2.52	2.21	2.61	1.24
135	2.82	2.41	2.41	2.12	2.81	1.28
150	3.02	2.54	3.01	2.69	2.98	2.10
165	3.09	2.39	3.12	2.79	2.99	2.21
180	3.15	2.86	3.26	2.92	3.60	2.20

TABLE 46. Effect of frozen storage on peroxide value in different species of fish and shellfish

Frozen Storage, days	Peroxide value, milli equivalents/100 g fish muscle					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.56	2.80	0.31	1.80	0.79	0.42
15	0.52	3.85	2.12	4.12	1.12	2.85
30	0.48	8.43	3.95	8.48	1.63	2.84
45	0.45	10.52	5.85	8.50	1.85	4.61
60	1.52	12.16	8.41	10.15	4.61	6.13
75	3.84	13.89	8.40	11.18	7.85	8.13
90	10.40	15.92	13.56	14.16	9.18	10.83
105	11.52	16.45	14.12	16.73	12.14	11.45
120	13.21	19.84	14.52	17.01	19.85	12.84
135	15.24	32.52	16.45	24.15	25.85	15.46
150	16.52	28.12	21.48	24.98	23.45	14.50
165	16.42	27.48	20.31	25.84	22.98	13.15
180	17.30	28.40	21.50	24.40	23.10	13.40

This was followed by prawn and mrigal. Least rise was found in mullet. Correlation coefficients between PV and period of frozen storage were worked out. Highly significant positive correlation ($P \leq 0.01$) was shown by all the species subjected to frozen storage. It shows that peroxide value of fish muscle is directly proportional to period of frozen storage.

Thiobarbituric acid value has been determined in the six species of fish and shellfish subjected to frozen storage. In all the species studied frozen storage caused an increase in TBA number. Substantial increase in TBA value was shown by tilapia followed by mullet. P. indicus also showed considerable rise in TBA value. Table 42 gives an idea about the linearily of the relation between TBA value and days of frozen storage in the six species of fish and shellfish studied. Highly significant positive correlation was seen with the species mullet, pearlspot, milk fish, tilapia and P. indicus. Although positive correlation was shown by the species mrigal, this was not found statistically significant.

Sensory evaluation of the samples were carried out at regular intervals during the period of frozen storage. Regular fall in various sensory scores of the samples were observed in all the species subjected to frozen storage. Fig. 18 shows steady decrease in overall acceptability score in the various species studied. Karl Pearson's correlation coefficients were worked out to establish the relationship between sensory score and storage period. Results are shown in Table 48. Highly significant negative correlations were observed in all the species subjected to frozen storage.

TABLE 47. Effect of frozen storage on Thiobarbituric acid number in the meat of various species of fish and shellfish .

Frozen Storage, days	TBA number, mg malonaldehyde/kg fish					
	Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
0	0.09	0.61	0.21	0.05	0.04	0.02
15	1.12	0.92	0.20	0.62	0.08	0.04
30	1.48	1.85	0.11	0.91	0.10	0.08
45	1.84	2.21	0.08	0.90	0.12	0.12
60	1.73	2.73	0.92	1.18	1.41	0.15
75	1.65	2.65	1.10	1.92	0.91	0.18
90	1.62	2.91	1.15	2.15	1.58	0.52
105	1.73	3.15	1.95	2.31	1.92	0.76
120	2.30	4.85	2.32	3.26	2.15	0.89
135	2.15	4.80	2.48	3.84	2.21	0.98
150	1.93	4.70	2.20	3.81	3.08	0.85
165	1.92	4.68	2.30	3.12	3.51	0.90
180	1.80	4.75	2.01	3.30	3.60	0.89

TABLE 48. Correlation coefficient matrix between sensory scores and storage period in the six species of fish/shellfish during frozen storage. Significance levels, * $P \leq 0.05$ ** $P \leq 0.01$

Sl. No.	Sensory Tests	Correlation coefficients (r)					
		Mrigal	Mullet	Pearlspot	Milkfish	Tilapia	P. indicus
1.	General appearance	0.831*	0.892*	0.923**	0.951**	0.891*	0.862*
2.	Odour score	0.938**	0.849*	0.681	0.971**	0.921**	0.869*
3.	Texture score	0.961**	0.921**	0.938**	0.961**	0.957**	0.815
4.	Flavour score	0.868*	0.831*	0.632	0.785	0.921**	0.867*
5.	Overall acceptability score	0.922**	0.977**	0.957**	0.957**	0.921**	0.976**

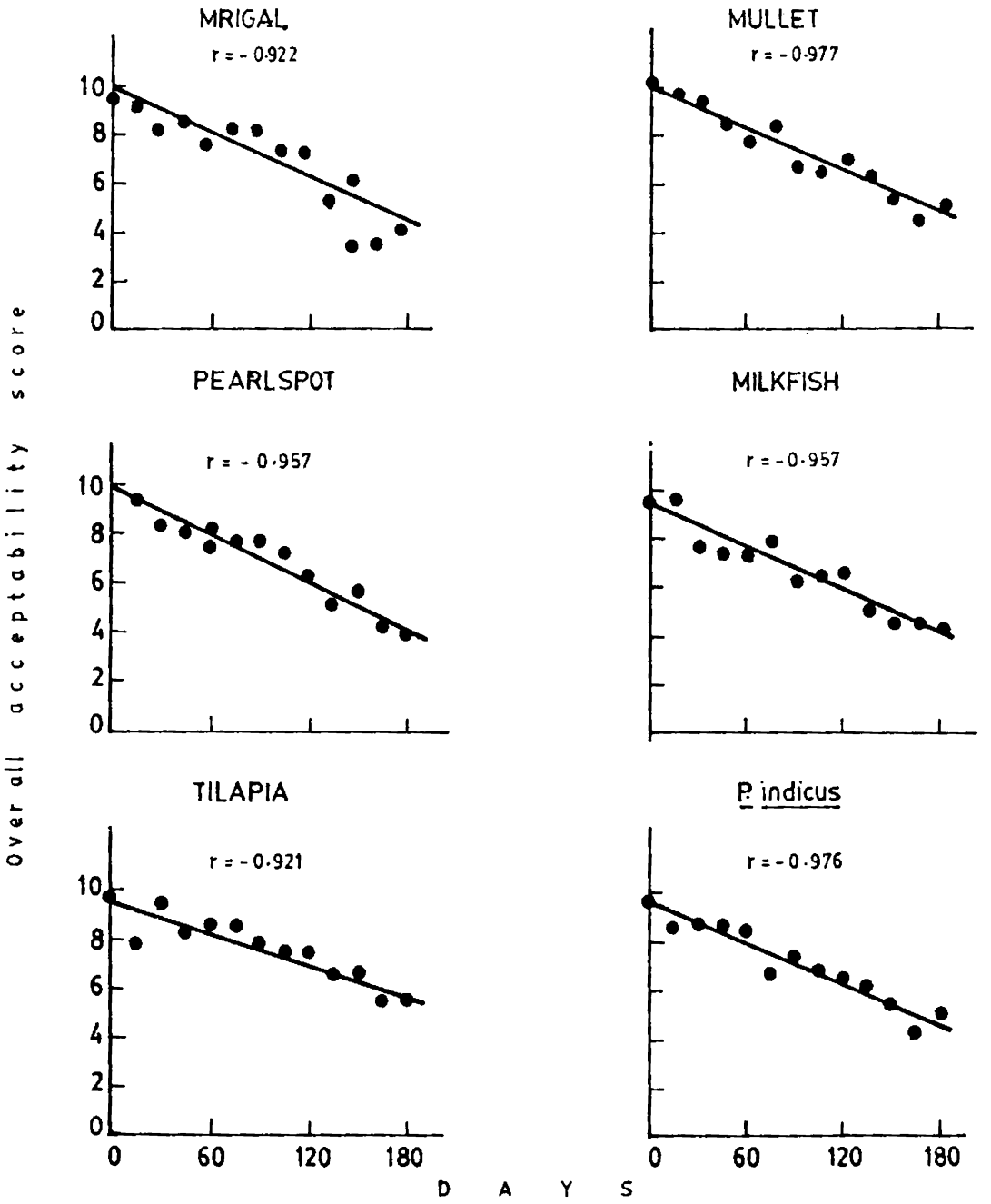

FIGURE 18. Effect of frozen storage on overall acceptability score in the six species of fish and shellfish. Correlation coefficients and regression lines showing the relationship between storage period and overall acceptability score are also shown in the figure.

4.2 DISCUSSION

The present study has yielded results enlightening the following aspects:

1. Effect of freezing and subsequent thawing on enzyme activity in press juice of fish muscles.
2. Enzyme activity in fish muscle subjected to prolonged cold storage.
3. Correlation between changes in enzyme activity and freshness indices in fish muscle subjected to cold storage.

4.2.1 EFFECT OF FREEZING AND SUBSEQUENT THAWING ON ENZYME ACTIVITY IN PRESS JUICE OF FISH MUSCLES

The study has shown that freezing and subsequent thawing cause remarkable increase in the activity of lipoamide reductase in the press juice of fish muscles. Among the different species studied, substantial increase in lipoamide reductase activity was observed with milkfish and P. indicus. Similarly freezing and thawing caused considerable rise in press juice-LDH and 5'AMP deaminase activity in the different species, especially pearlspot and tilapia. Ca^{2+} ATPase activity also followed similar trend. However the results were not as significant as in the case of other enzymes.

Any test composed of a one time determination of enzyme activity will be a useful tool in differentiating fresh fish from that which have been frozen and thawed only when such a test will be supported with data on the variations in enzyme activity in fish muscles within the species during different seasons. Since the above observations reported in this thesis were recorded during the main experiment on frozen storage studies, obtaining such data were beyond the scope of this work. Nevertheless the very high values of

lipoamide reductase activity observed in frozen thawed muscles of the species mrigal, milkfish, tilapia and P. indicus indicate that lipoamide reductase activity may be employed as a test for detecting whether fish have been frozen and thawed.

Hamm and Kormandy (1969) reported similarly increase in glutamate oxaloacetic transaminase activity in mammalian muscle on freezing and thawing. However this could not be employed with carp since the mitochondria were already destroyed to a high degree during normal storage of carp fillets in ice (Rehbein et al, 1978).

Similarly Gould (1971) determined an iso-enzymic form of malic dehydrogenase (E.C. 1.1.1.40) in press juice of frozen and thawed fillets. The determination of this isoenzyme in press juice was applied successfully to differentiate thawed shucked oysters from fresh ones but the authors did not obtain reliable results with fish (Gould, 1968).

Rehbein et al (1978) determined the specific activities of the lysosomal enzymes αglucosidase and βN-acetyl glucosaminidases in press juices and extracts from fresh and frozen thawed fillets of cod, saithe, red fish and haddock. With all the above species, specific activities of the enzymes in press juice increased with freezing and thawing. Barbagli and Crescenzi (1981) have observed that freezing and thawing of animal tissues cause a remarkable release of cytochrome oxidase from mitochondria.

The release and subsequent increase in specific activity of certain enzymes in sarcoplasm have been suggested by many workers as a method to distinguish fresh animal tissues from frozen and thawed. Chhatbar and Velankar

(1977) observed that freezing and thawing of four species of tropical fish lead to an increase in the total activity of aspartate aminotransferase in tissue fluid due to the release of bound form of mitochondial enzyme. Cattaneo et al (1982) reports that the specific activity of the lysosomal enzyme αglucosidase in press juice from trouts increased significantly on freezing and thawing. Demmer and Werkmeister (1985) identified fresh meat from frozen and thawed meat based on the ratio of βhydroxy acyl CoA dehydrogenase activity in the press juice of muscle to the total extracted enzyme activity.

Yoshioka (1985) reports a non enzymatic method to distinguish freeze thawed fish from fresh fish. He has employed the hematocrit value as the destruction index of red blood cells in the unfrozen and freeze thawed fish. The experimental results obtained by carp indicated that the changes in hematocrit values during the unfrozen and frozen storage reflected well the destruction degrees of red blood cells.

The process of freezing animal tissues leads to the disruption of organelles like mitochondria, lysosomes etc. releasing into the cell saps the enzymes bound to these structures. Reportedly this causes an increase in enzyme activity in the press juice of frozen thawed fish muscle. A large number of freeze-thawed fish have been often sold without being distinguished from fresh fish at the final stage of marketing. It is in this context, development of a simple method to distinguish fresh fish from frozen thawed fish becomes all the more important.

4.2.2 ENZYME ACTIVITY IN FISH MUSCLE SUBJECTED TO PROLONGED COLD STORAGE

In the study reported in this thesis, enzyme assays were carried out in six

species of fish/shellfish during the period of frozen storage. On prolonged cold storage a steady decrease in the activity of the enzymes Ca^{2+} ATPase, and LDH were observed in most of the species studied. Although decrease in 5'AMP deaminase activity was observed in some of the species this was not significant in the rest of the samples. The results pertaining to the above enzymes are discussed separately.

4.2.3 CA^{2+} ATPase ACTIVITY

Ca^{2+} ATPase activity in all the six species except P. indicus registered significant fall with period of frozen storage. Significant or highly significant linear relations between Ca^{2+} ATPase activity and other freshness tests were shown by all the species of fish. The shellfish (P. indicus) gave correlation values which were not significant.

Based on the results of sensory evaluation and other physico chemical and biochemical indices of freshness, critical values of Ca^{2+} ATPase are given below in the frozen stored samples. A lower values of Ca^{2+} ATPase in frozen stored fish/shellfish than that shown below will indicate that the sample has crossed the limit of consumer acceptability.

Fish/prawn	Ca^{2+} ATPase specific activity, (μmole Pi/min/mg protein)
Mrigal	0.032
Mullet	0.011
Pearlspot	0.088
Milkfish	0.038
Tilapia	0.072

In the case of the shellfish P. indicus no significant correlation was observed between Ca^{2+} ATPase activity and period of frozen storage.

The results are in agreement with the observations made by Connel (1960) on ATPase activity in fish stored at $-14^{o}C$ & $-22^{o}C$. Similarly Jiang (1977) reported that Ca^{2+} ATPase activity of mullet stored at $-20^{o}C$ for a period of one year decreased markedly. Significant negative correlation was observed between Ca^{2+} ATPase activity and frozen storage period in the above species. Myofibrillar Ca^{2+} ATPase activity gives an index of the extent of freeze denaturation of myofibrillar proteins (Fukuda et al, 1984).

Initial increase in Ca^{2+} ATPase activity as seen in some of the samples subjected to frozen storage is considered to be caused by the increased affinity of actin-myosin interaction.

4.2.4 ENZYME SPECIFIC ACTIVITY AT SUB-ZERO TEMPERATURES

Rate constants k of chemical reactions are temperature dependent and obey the Arrhenius relationship

$$k = A \exp\left(-\frac{E}{RT}\right)$$

where A is a preexponential factor and E is the activation energy.

This expression can be written

$$\frac{d\,(\ln k)}{dt} = \frac{E}{RT^2}$$

When the logarithm of the velocity of an enzyme reaction at sub-zero temperatures is plotted against 1/T sharp breaks in the Arrhenius plots

were observed. It was pointed out that this was caused by reversible formation of a catalytically inactive enzyme the concentration of which increase sharply with decrease in temperature. Considering that the high temperature denaturation of an enzyme unfolds its conformation to such an extent that the specific structure of the active centre is lost, the reverse process may be an important factor in the formation of a catalytically inactive enzyme at low temperature (Douzou, 1977). The reactive configuration of the enzyme would be possessed in only a relative narrow temperature range, being lost at both high and low temperatures.

4.2.5 FREEZE DENATURATION OF ENZYME

ATPase activity of actomyosin isolated from frozen stored fish meat lowers proportionately to the length of storage period. Myosin molecules are formed from the head part with amorphous structure and cylindrical part with α- helix conformation. Since the site of ATPase activity exists in the head part (HMM) lowering of the ATPase activity proves that the conformation of the site has been deformed by causing myosin molecules to aggregate during frozen storage.

Dissociation into subunits has been reported to be the cause of inactivation at subzero temperatures of enzymes which are oligomeric proteins, i.e., proteins which have two or more subunits. This has been reported in the case of ATPase of beef heart mitochondria, erythrocyte glucose-6-phosphate dehydrogenase, soluble ATPase which loses its activity at $4^{o}C$ and does not undergo a ractivation on warming (Douzou, 1977).

4.2.6 LACTATE DEHYDROGENASE ACTIVITY

The present study has shown high correlation of LDH activity with storage period with the species mrigal, mullet, pearlspot, milkfish and tilapia. LDH activity significantly decreased with period of frozen storage. However correlation was not significant in the case of P. indicus. Similar trend was shown by LDH in press juice of fish muscles. However significant correlation between LDH activity both in fish muscle extract and press juice and freshness tests were seen only with a few species studied.

Based on the results obtained critical values of LDH activity are estimated, below which the frozen fish samples will be unacceptable organoleptically.

Fish/prawn	LDH specific activity, (NADH μmoles/min/mg protein)
Mrigal	256.1
Mullet	148.7
Pearlspot	174.1
Tilapia	204.3
Milkfish	434.1

LDH activity in P. indicus disappeared totally at the end of 120th day of frozen storage while the fish was still acceptable organoleptically.

Very little work has been reported in the past on LDH assay in fish muscle subjected to frozen storage. Sudhershana- (1981) carried out a study of the isozyme pattern of tissues of L. rohita stored at -20^{o}C for 31 days and found no major difference in isozyme pattern.

Some studies have been reported on malate and glutamate dehydrogeneses in fish muscle subjected to frozen storage. A progressive decline in malate dehydrogenase activity was shown by the muscle of cod, pollock and dab when stored at $-7^{o}C$ for a period of 5 months (Gould 1964). Hamm & El-Badawi (1984) observed a reduction in total extractable activity of glutamate dehydrogenase in mammalian muscle stored at $-20^{o}C$ for a period of three months.

The fall in LDH activity during frozen storage can be caused by freeze denaturation of the enzyme. Fish LDH exhibit temperature stabilities of the same order as those from warm blooded mammels (Tarr, 1966).

4.2.7 EFFECT OF LIPID HYDROLYSIS ON ENZYME ACTIVITY

This study has revealed highly significant correlation between enzyme activity and FFA content of muscle. Increase in FFA content of muscle has caused decrease in the activity of the enzymes, Ca^{2+} ATPase, lipoamide reductase and to some extent LDH. Although negative correlation was shown by 5'AMP deaminase, this was significant only with two species.

Earlier reports also indicate that FFA accumulate with prolonged frozen storage. The maximum rate of lipid hydrolysis in many fish species (cod, sole, halibut, and many gadoid species) was found at temperatures just below freezing, at $-4^{o}C$ (Lovern and Olley, 1962). It was observed that the decrease in protein extractability followed more or less the same pattern of FFA accumulation (Shenouda, 1980). Highly significant negative

correlation between Ca^{2+} ATPase activity and FFA content has been established in the present study which is in agreement with reports from other workers.

King et al (1962) studied the factors that influence FFA - myofibrillar protein interaction. Free fatty acids are believed to attack primarily the myofibrillar proteins. Their binding to sarcoplasmic proteins is not excluded but apparently is less effective in in solubilising them.

It has been observed in the case of cod that protein denaturation and phospholipid break down or FFA production run parallel (Olley & Lovern, 1960; Dyer & Fraser 1959). Olley et al (1962) has shown a close similarity between protein denaturation in cod and FFA production due to phospholipase activity at various cold storage temperature. Rao (1984) reported that when rohu (Labeo rohita) actomyosin was mixed with oleic acid and frozen stored a decrease in percent Ca^{2+} ATPase activity was observed, thereby suggesting, the role of FFA in the denaturation of rohu actomyosin. However Olley et al (1962) has reported that the deduction that the two phenomena are related can obviously not be extended to other species. Connel (1960) considers that the different responses of various species of fish to freezing and cold storage are due to intrinsic differences in the stability of the muscle myosins. Aggregation of cod myosin, for example, proceeds at twice the rate of that of sole myosin.

The effect of FFA on the myofibrillar structure as revealed by electron microscopy (Jarenback and Liljemark, 1975) showed that low levels of FFA induced aggregation of the extracted proteins, but the fibrils retained a

great deal of their original shape. The mechanism of FFA myofibrillar protein interaction occurs primarily through secondary forces - electrostatic, vander waals, hydrogen and hydrophobic forces.

4.2.8 ENZYME ACTIVITY AND ∝AMINO NITROGEN CONTENT OF MUSCLE

∝amino nitrogen content in the species mrigal, mullet, pearlspot and milkfish decreased with storage period. Highly significant negative correlation was observed between storage period and ∝amino nitrogen content. Similarly positive correlation was found between the activity of the enzymes Ca^{2+} ATPase and LDH and ∝amino nitrogen content.

However prawn muscle is an exception to this. With the species P.indicus ∝amino nitrogen content was found to increase up to 90 days frozen storage. Further storage caused progressive decrease in ∝amino nitrogen content. No significant correlation between activity of the enzymes such as Ca^{2+} ATPase and LDH and ∝amino nitrogen content was observed in P. indicus. The steady rise in ∝amino nitrogen content in prawn was proof for the occurrence of definite though small proteolytic reactions. Increased deamination reaction on prolonged cold storage caused fall in ∝amino nitrogen content.

4.2.9 CORRELATION BETWEEN ENZYME ACTIVITY AND TOTAL VOLATILE NITROGEN CONTENT OF MUSCLE

TVN content of fish muscle was found to increase with increase in period of frozen storage. Karl Pearson's correlation coefficient values show highly significant positive correlation between the two variables. Similar

observations were made by Chakrabarti (1984) on C. mrigala and Hale and Waters (1981) and Govindan (1982) on shrimp.

TVN values showed highly significant negative correlation with specific activities of both Ca^{2+} ATPase and LDH in fish muscle extract. This study on Ca^{2+} ATPase activity of myofibrillar proteins in comparison with TVN values shows that the former is useful in evaluating the quality of minced fish meat in the refrigeration process.

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CHAPTER 5

COLD SHOCK REACTIONS IN TROPICAL FISHES

5.1 INTRODUCTION

Tropical fish have been found to exhibit a cold shock reaction similar to that of rigor mortis. Locker and Hagyard (1963) were the first to observe a cold shortening in terrestrial animal muscle. Curran et al (1986) in an extensive study conducted recently have shown that in tilapia (Oreochromis aureus/Niloticus hybrid) cold shock stiffening occurred after 2 hr at $0^{o}C$ and after 7 hr at $22^{o}C$. It was also reported by Disney et al that iced tilapia exhibited a rigor like shortening which developed within one hour. Un-iced fish however did not enter rigor mortis until 2-5 hr after capture.

Rigor in a decisive way predetermines the shelf life of fresh fish. When rigor is extended in time either through a delay in its on set or by its duration, this correspondingly defers the attack by the bacteria of spoilage (Curran et al ,1986). On the other hand an intense contraction during rigor mortis may affect the connective tissue and increase gaping in fish. Also stiffening caused by rigor mortis poses problem in filleting of fish especially when carried out at sea. Results of a comparative study on rigor mortis and cold shock reactions will significantly contribute towards improving the handling practices of many species of tropical fishes.

The objective of this study was to investigate the problem of cold shock reactions in several species of marine, fresh water and brackish water fishes and to compare cold shortening with rigor mortis set in fish at room temperature.

5.2 MATERIALS AND METHODS

Representative samples from fresh water, brackish water and marine fishes were used for the study. Fresh water fishes such as mrigal, rohu and silver carp were collected from the fresh water ponds maintained by the College of Fisheries, Panangad. Fishes were caught by cast net and brought alive to the laboratory.

Brackish water fishes: Fishes such as mullet, tilapia and milk fish were harvested using dragnet from the brackish water ponds maintained by College of Fisheries,Panangad. Samples were brought alive to the laboratory.

Marine fishes: Marine fishes were collected from the Chinese nets operated at Fort Cochin and also from trawlnet operated by the vessel Matsya-I. In the latter case ice storage studies were conducted on board the vessel.

Sampling: Fishes belonging to the same age group were employed for the study. Analysis were conducted in duplicate and the reported values are mean values.

Ice storage of fish was carried out by mixing fish with ice in the proportion 1:2 in an ice box. The temperature $15^{o}C$ was achieved in the lower chamber of a refrigerator. Temperature was accurately maintained with the help of a freezer temperature monitor. Room temperature was $28 \pm 1^{o}C$. The temperature $37^{o}C$ was obtained in an incubator provided with a thermostat.

Determination of pH: This was carried out on a 2:1 water fish homogenate using a glass electrode.

ATpase assay: This was determined as per the method described by Noguchi

and Matsumoto (1970). Inorganic phosphorous in the reaction mixture was determined by the method of Fiske & Subbarow (1925). ATPase activity was reported as micromole Pi/min/mg protein.

Determination of Glycogen: Glycogen content of fish tissue was determined as per the proceedure described by Hassid & Abraham (1957).

Stiffening: A simple method described by curran et al (1986) was used for the determination of stiffening. In this method the sag of the tail was noted when the fish are held vertically by the head with the tail pointing upwards.

The assessments were carried out at intervals of 10 minutes following death. Curran described five stages of stiffening viz. (1) flaccid, (2) tail bent half-way, (3) tail bent slighty, (4) tail straight, and (5) whole fish very rigid. In the present study, fish was considered in rigor when it was in stage (4) tail straight.

5.3 RESULTS AND DISCUSSION

Table49 illustrates the time taken by different species of fish for undergoing cold stiffening at $0^{o}C$ and rigor mortis at room temperature. Stiffening occured in fish more rapidly at $0^{o}C$ than at room temperature. Among the fresh water species this phenomenon was more prominent in mrigal. In iced mrigal, stiffening occured within 10 minutes after death where as in samples kept at room temperature, the time of on set of rigor was 120 minutes.

Cold shock reactions in brackish water fishes considerably varied between species. Onset of stiffening was significantly advanced in mullet stored in ice than exposed to room temperature. Iced storage resulted in rapid onset

TABLE 49. Cold shortening in different species of tropical fishes

No.	Species	No. of fishes examined	Length Cms	Weight g	Time taken for onset of Stiffening, mins	
					Iced	Room Temp.
	Fresh Water Fishes					
1.	Mrigal (Cirrhinus mrigala)	6	21	120	10	120
2.	Rohu (Labeo rohita)	6	20	100	40	120
3.	Silver carp (Hypophthalmichthys molitrix)	4	36	573	90	170
4.	Common carp (Ciprinus carpia)	6	20	170	30	60
	Brackish water fishes					
5.	Mullet (Liza parsia)	6	24	155	30	60
6.	Cat fish (Tachysurus Sp)	6	25	120	40	60
7.	Therapon (Therapon jarbua)	6	12	70	40	60
8.	Long rayed silver biddy (Genres filamentosus)	8	16	62	20	30
9.	Spotted butter fish (Scatophagus argus)	6	14	83	30	50
	Marine fishes					
10.	Six banded trevally (Caranx sexfasciatus)	8	12	12	20	30
11.	Parava (L. lactarius)	6	17	75	10	70
12.	Malabar achovy (Thrissocles malabaricus)	8	14	40	10	40

of stiffening in all the species of marine fish studied. This effect was greatest in Parava (L. lactarius).

In order to understand the effect of different temperatures on the onset of stiffening in tropical fishes an experiment was conducted by placing the same species under different temperatures and noting the time taken for the onset of rigor. Results are given in Table 50. Rapid stiffening was observed in all the species of fish stored at 37°C. Stiffening was observed within minutes of exposure to 37°C. Further studies are required to reveal the true nature of this phenomenon of thermal shock reactions in tropical fishes. More or less similar reaction was observed in samples exposed to 0°C. Table also shows the time taken for the onset of stiffening in different species of fish exposed to 15°C. Time taken for rigor setting was greatest in those samples exposed to room temperature.

pH: Muscle pH was determined in fresh fish and also in fish at the time of onset of stiffening at 0°C and rigor mortis at room temperature. Muscle pH was highest in fresh fish. A slight lowering of pH was observed in fish that have undergone stiffening at 0°C. pH further lowered at the onset of rigor mortis in samples stored at room temperature. This pattern of change in pH is in good agreement with the results obtained for white fish muscle at the onset of rigor mortis (Manohar 1971).

Love (1980) reported that the pH of mullet and silver carp muscle was above neutrality while the fish was still alive. After the death of fish

TABLE 50. Effect of different temperatures on the onsets of rigor mortis in fish.

Temperature	Time in minutes			
	Rohu	Milkfish	Mullet	Silver carp
$0^{o}C$	40	20	33	90
$15^{o}C$	100	10	35	70
Room Temp. ($28^{o} \pm 1$)	120	90	40	150
$37^{o}C$	30	15	30	60

some of the residual carbohydrate in the muscle is converted anaerobically to lactic acid which together with any lactic acid already present from struggling before death causes the pH to fall. It takes about 15 hours at $0^{o}C$ after death for the pH to reach its minimum value (MacCallum et al, 1967). The fall in pH is proportional to the amount of lactic acid present but not necessarily to the initial amount of glycogen present in the muscle some of which is converted hydrolytically to glucose which does not influence the pH (Kida and Tamato, 1969). The study shows that in cold shortening stiffening occurs without significant fall in pH. This distinguishes cold shortening from that of rigor mortis.

ATPase activity: ATPase activity of fresh fish muscle was compared with that of muscle at the time of cold shortening and the onset of rigor mortis. ATPase activity determination gives a measure of the extent of denaturation the myofibrillar protein has undergone. The results are shown in Table 5. Muscle ATPase activity at the time of stiffening in fishes stored in ice was found higher than that in muscle of fish immediately after death. Higher ATPase activity was found in fish kept at room temperature. In mullet, milk fish and silver carp similar result was observed. Rigor mortis in fish is induced with a decrease in ATP level of muscle (Partmann, 1965). The intensity of rigor mortis was found dependent upon the amounts of ATP decomposed per unit time (Hiltz et al, 1974). However, in the case of cold stiffening it was observed that shortening occured while about 40% ATP still remained in the muscle. Thus cold shortening was found to more closely resemble thaw rigor than normal post rigor changes. The ATP level remains rather constant during

TABLE 51. Biochemical changes in the muscle of fish immediately after death and at the time of onset of rigor mortis

Fish	Storage condition	pH	ATPase activity	Glycogen mg/g tissue
Mullet	Immediately after death	7.38	0.365	3.50
	$0^{o}C$	7.20	0.384	1.68
	Room Temp.	6.82	0.456	1.44
Chanos	Immediately after death	6.26	0.409	8.95
	$0^{o}C$	6.24	0.460	7.87
	Room Temp.	5.90	0.429	7.10
Silver Carp	Immediately after death	7.12	0.259	6.12
	$0^{o}C$	7.01	0.271	6.04
	Room Temp.	6.81	0.292	5.22

cold stiffening because of a fast resynthesis of ATP from adenosine diphosphate by the glycolytic process (Honikel & Hamm, 1978).

Glycogen content: Table 5i also shows the muscle glycogen content in fishes in the 3 different conditions described above. Highest glycogen content was observed in muscles of fish immediately after death. Lowering of glycogen was observed in muscles of fishes stored at $0^{o}C$ and at room temperature. Manohar (1971) has reported similar rapid decrease in glycogen content of white fish muscle held in ice.

Cold shock phenomenon is explained by the influence of temperature on the membrane system of the sarcoplasmic retiiculum. Below about $15^{o}C$ decreasing temperature causes an increasing inactivation of the ATP driven calcium pump of the sarcoplasmic reticulum which transports Ca^{2+} ions from the sarcoplasma into the sarcoplasmic reticulum. Therefore Ca^{2+} ions are released from the sarco tubular system, they activate the myosin ATPase and consequently initiate the onset of stiffening (Honikel & Hamm, 1978)

SUMMARY AND CONCLUSION

This thesis reports the findings of a study on the potentiality of certain endogenous enzyme systems as tools of quality control of fish and shellfish. Activity determinations of the four enzymes viz., Ca^{2+} ATPase, lipoamide reductase, lactate dehydrogenase and 5'AMP deaminase were carried out in the species mrigal, mullet, pearlspot, milkfish, tilapia and P. indicus. Stability of the enzymes were studied in fish and shellfish during the period of iced and frozen storage. In addition to this, the effect of freezing and thawing on the activity of the enzymes in press juice of muscles were determined. Results of a study on cold shock reactions in tropical fishes are also given in this thesis.

Ice storage of fish and shellfish caused considerable and significant loss in the activities of the cytoplasmic enzymes lactate dehydrogenase and 5'AMP deaminase in the muscles of fish and shellfish. Critical values of the activities of LDH and 5'AMP deaminase in the muscle extract of ice stored fish and shellfish were computed based on the result obtained for sensory evaluation and other tests evaluating freshness of fish. Fishes having enzyme activity values below the critical values indicated here are considered unacceptable.

In the species such as mrigal, mullet, pearlspot and tilapia stored in ice, critical values of LDH specific activities (expressed as NADH μ mole/min/mg protein) ranged from 312.1 to 545.0. Limiting values of 5'AMP deaminase

specific activity (expressed as units/mg protein) ranged from 1.38 - 2.92. High limiting value of LDH specific activity was observed in the species milkfish during ice storage. 5'AMP deaminase activity in the species milkfish decreased to 0.03 at the end of 6 days storage in ice and at the end of 9 days storage total loss in enzyme activity occured. Limiting values of specific activities of the enzymes LDH and 5'AMP deaminase in the species P. indicus were 21.7 NADH/U moles/min/mg protein and 0.07 units/mg protein respectively.

Although loss in activities of the enzymes Ca^{2+} ATPase and lipoamide reductase were observed in a few species subjected to storage in ice, this trend was not consistent and significant in the remaining species.

Highly significant correlations were observed between activities of the enzymes LDH and 5'AMP deaminase in ice stored fish and shellfish and freshness indices such as total volatile nitrogen, ∝amino-nitrogen, free fatty acid and overall acceptability score. The phenomenon of leaching out of the water soluble nitrogen and ∝amino nitrogen from the muscle into the ice melt water has been reported. Highly significant positive correlations were observed between fall in activities of the enzymes LDH and 5'AMP deaminase and freshness test values such as ∝amino nitrogen and total volatile nitrogen.

Effect of freezing and thawing on enzyme activity in press juice of muscles were determined. This study shows that the substantial increase in the activity of the enzyme lipoamide reductase in the press juice of muscles on freezing and thawing can be employed in distinguishing fresh

fish from that which has been frozen and thawed. The increase in activity is caused by releasing of the mitochondrial enzyme on freezing and thawing. Slight increase in the activities of the enzymes LDH and 5'AMP deaminase in press juice of muscles were also observed on freezing and thawing. However, this was not so significant as in the above case.

Prolonged cold storage of fish and shellfish resulted in a steady decrease in the activities of the enzymes Ca^{2+} ATPase and lactate dehydrogenase. Although decrease in 5'AMP deaminase activity was observed in some of the species, this trend was not significant in the remaining samples.

In the frozen stored fishes critical values of enzyme activities are determined, below which the samples will be considered unacceptable. These values were computed based on the corresponding sensory score and results of freshness tests. In the case of fish muscle Ca^{2+} ATPase, limiting values in the range of 0.011 - 0.088 μ mole Pi/min/mg protein has been observed for all the five species of fishes studied. In most species of fishes studied, limiting values of LDH activity ranged from 174.1-204.3 NADH μmole/min/mg protein. In the species, P. indicus, disappearance of LDH activity could be taken as an early sign of loss in freshness.

This study has shown highly significant negative correlation between free fatty acid content in frozen stored fish muscle and activities of the enzymes Ca^{2+} ATPase, and to some extend LDH. The presence of FFA is reported to cause aggregation of myofibrillar proteins and can be one of the causes of fall in enzyme activity. Another cause for fall in

enzyme activity can be denaturation due to increase in solute concentration caused by freezing which in turn will lead to changes in ionic strength and pH.

A study was conducted on cold shock reactions in several species of fresh water, brackish water and marine species of fish. When compared to storage at room temperature, chilling resulted in rapid onset of stiffening in the various species studied. A comparative study on the biochemical characteristics of cold shock reactions at $0^{o}C$ and rigor mortis at room temperature was carried out to find out the subtle differences between the two phenomena. A definite lowering in muscle pH was found associated with stiffening at $0^{o}C$. Muscle glycogen content has also undergone similar pattern of change. Muscle ATPase activities was determined to find out the extent of denaturation of myofibrillar proteins. Although ATPase activity at the time of onset of stiffening at $0^{o}C$ was higher than activity at the time of death, it was found lower than in fish muscle undergoing rigor at room temperature. Studies on the effect of exposure to different temperatures on the onset of stiffening in tropical fishes have shown an intense thermal shock in fishes exposed to $37^{o}C$.

Cold shortening was found to more closely resemble thaw rigor than normal post rigor changes. This phenomenon is explained by the influence of lowering of temperature on the membrane system of the sarcoplasmic reticulum triggering a series of changes which ultimately initiate the onset of stiffening.

Enzymes such as Ca^{2+} ATPase, LDH and AMP deaminase have shown potentialities in the determination of freshness of fish and shellfish. Many of the chemical tests for freshness are based on the quantitative determination of one or a group of substance formed as a result of post mortem spoilage reactions. One of the problems associated with these tests is that by the time a detectable concentration of these substances are formed the product might have undergone considerable loss in freshness. This problem can be overcome by employing enzyme assay.

One of the limitations of employing enzyme assays in testing the freshness of fish and shellfish is the variation in the initial level of enzyme activity in different species. The critical values of enzyme activity depend on the initial levels of activity. Determinations of variations in enzyme activity level in the same species during different seasons are prerequisites in developing any enzyme assay as a freshness test. However, the above work was beyond the scope of this thesis and hence not attempted.

Interesting results were obtained in the study conducted on cold shock reactions in various species of tropical fishes. Chilling resulted in a rapid onset of stiffering in the various species studied. This contradicts earlier observation that icing delays the onset of rigor mortis. It will be of interest to the fish processor to know the effect of cold shock on keeping qualities of fish. With this in mind an investigation was taken up to study the biochemical characteristics of cold shortening. It was observed that cold shortening resembles more closely to thaw rigor than normal post rigor changes.

Commercial application of enzyme assay as a freshness test depends on its simplicity in adoption for routine work. Assay procedures of most of the enzymes studied in this work are complex requiring sophisticated equipments. Further work may be taken up for developing simple assay techniques for these enzymes studied such as LDH, Ca^{2+} ATPase, and 5'AMP deaminase which have been found useful in testing the freshness of fish.

Another area requiring future investigation is the phenomenon of thermal shock observed in many species of fishes exposed to $30^{o}C$. It will be interesting for the fisheries scientist to know the bio-chemical differences between thermal shock and cold shock reactions and also the effect of these phenomena on post mortem deteriorative changes in fish. Hence, further research on this topic is recommended.

It may be concluded that in this study major stress was given for screening some of the muscle enzymes for their application in testing the freshness of fish and shellfish. This is an area promising immense potentialities for future investigations. The problem of variation in enzyme activity level in each species can be overcome by recommending critical values of enzyme specific activity for various species. With rapid progress in analytical techniques, it is not unreasonable to expect the development of simple enzyme assay techniques for routine application. It is believed that this study will stimulate research in this direction and new techniques and new insight will lead to greater achievements in this area.

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APPENDIX - I

LIST OF PUBLICATIONS

I. Research Papers

1. Damodaran Nambudiri D., and K. Venugopal, (1978). A comparative evaluation of some of the objective methods and organoleptic assessment for testing the freshness of iced prawn. Proceedings of the Seminar on Quality Control of Processed Foods, AFST (I), Trivandrum, P. 95.

2. Damodaran Nambudiri, D (1980) Mixed culture fermentation as a predominant biological phenomenon in the production of fermented fish products, Proceedings of the symposium or Coastal Aquaculture, MBAI, Cochin, P. 176.

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9. Damodaran Nambudiri, D and Gopakumar, K. 1987. Cold shock reactions in tropical fish. J. Food Sci. Technol. 25. (under print).

II. Book Published

1. Damodaran Nambudiri, D. 'Analytical Manual of Fish & Fishery Products' Kerala Agricultural University Press, Trichur, 1985.

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