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Microbial Enzymatic Activity in some Marine Sites of the North Mediterranean sea Coast of Egypt

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ABSTRACT

Microbial enzymatic activities of α- Amylase and β- Galactosidase enzymes were measured seasonally in marine water samples that were collected from two sites located at the south Levantine Sea basin of the Mediterranean Sea of Egypt, Port Fouad city in the east and Alexandria city in the west during four successive seasons from autumn 2014 to summer 2015. Determination of microbial enzymatic activities were carried out by determining reducing sugars using DNS reagent. This research provided a newly applied method for the determination of microbial enzymatic activities in natural open water system, the Mediterranean Sea. The applied methodology involved using marine water samples as the source of crude enzyme , eliminating the difficulties of extracting an enzyme from specific microorganism as well as avoiding the adjustment of aseptically required conditions for the inoculation and incubation of the desired microorganism in culture media tube under the laboratory conditions while providing almost natural conditions for the microbial community represented in water samples. The water samples were chemically analyzed to determine the effect of certain metal ions concentrations on the enzymatic activities of the microbial α- Amylase and β- Galactosidase enzymes. The results showed that though water samples were collected from the same marine source yet, spatial variations occurred between the two sampling sites and seasonal variations at the same sampling sites which were attributed to the naturally occurring environmental variations during the different seasons of the year.

Keywords : α-Amylase, β-Galactosidase, Bacteria.

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INTRODUCTION

Polymers like polysaccharides are not directly accessible for marine bacterioplankton, because bacterial cells cannot take up molecules larger than 600–800 Da[1, 2]**.** Therefore, bacterial polysaccharide turnover is controlled by two steps, the extracellular enzymatic cleavage of polymers and the subsequent uptake of hydrolysates. Among extracellular enzymes involved in polysaccharide breakdown, glucosidases are known to be ubiquitously present in seawater[3]. Glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds to liberate monosaccharaides and oligosaccharides of lower molecular weights. These enzymes are widely distributed in nature and found in all organisms and now they are known as Amylases. α-Amylase is an endo-acting enzyme which hydrolyses linkages in the starch polymer chain randomly, leading to the generation of linear and branched oligosaccharides[4].

The lactose-hydrolyzing enzyme, β-Galactosidase; catalyzes hydrolysis of lactose into glucose and galactose. The possible sources of the enzyme include bacteria, yeasts (intracellular enzyme) and fungi, among them bacterial sources are preferable because of ease of fermentation, high activities of the enzyme and good stability[5, 6].α-Amylase and β-Galactosidase enzymes are found in a wide variety of bacterial strains and their high activity and stability[7].

Bacteriological evidence indicating that gram negative bacteria with multilayered cell walls release little of the periplasmic enzymes, while gram positive bacteria lacking one or more cell wall layers tend to release enzymes extracellularly. The frequency of direct secretion of extracellular enzymes into the surrounding environment by the intact living gram negative cells is significantly lower in comparison to that of gram positive bacteria[8]. Conditions in the aquatic environments are unfavorable for extracellular enzymes for many reasons includingthe very low concentrations; high variability of many substrates, many substrates may be insoluble and exist in associations with other compounds; the extracellular enzyme itself might separate from the maternal cell and bind to suspended particles or it might be exposed to a variety of inhibitors present in seawater and the extracellular enzyme may also be denaturated by physical and chemical factors in the aquatic environments or hydrolyzed by proteases[8].

For the extracellular enzyme to be of great benefit for its organism it should avoid the destruction and bind with its substrate under appropriate physical and chemical conditions. Various aquatic microorganisms produce extracellular enzymes capable of reacting with many polymeric substrates and the microbial growth is dependent on the products of the extracellular enzymatic reactions[9, 10, 11].

Enzymatic activities of certain bacterial species under controlled cultural media conditions of temperature, pH, concentrations of sodium chloride ions and other parameters were measured in other literatures thus in this research the idea was to determine the enzymatic activity of α-Amylase and β-Galactosidase in the raw water samples collected from marine environmental sources under the environmental conditions and the enzymatically required conditions.

MATERIALS AND METHOD

Sampling sites

Two cities located at the eastern and western coasts of the south Levantine Sea basin of the Mediterranean Sea of Egypt were chose as sampling sites represented in Port Fouad city with meridians of 31°14'56" north and latitudes of 32°19'39"east and Alexandria city with meridians of 31°12'22" north and latitudes of 29°53'35" east.

Sampling

Water samples were collected seasonally for four successive seasons during the seasonal duration of autumn 2014 to summer 2015 from the sandy beaches of Port Fouad city and Alexandria city whereas composite surface water samples were collected in autoclavable sterilized glass jars of 1L capacity; the collected samples were transferred to the laboratory in ice box at 4°C where they were mixed well again for more accurate composite samples.

Enzymatic analysis

α- Amylase and β-Galactosidase were performed for water samples that were collected from the two sampling sites, considering these samples as the sources of microbial populations and sources of crude enzymes being tested.

Determination of glucose standard curve

2 mL of 3, 5-di-nitro salicylic acid reagent (DNS) were added to 1mL of each different glucose concentration of (0.1, 0.3, 0.5, 1, 1.5 and 2 mg/ml) and 1mL of 1 M citrate phosphate buffer pH 6.4 were added into each of the reaction tubes. The mixtures were boiled in water bath for 5 minutes and cooled under tap water then absorbance was measured at 540 nm using UNICO 2100 spectrophotometer. According to the Absorbance and concentrations, the glucose standard curve and regression equation were established[12, 13]. One unit (U/mL) of enzyme activity was defined as: the amount of α-Amylase and β-Galactosidase required to liberate 1 μmol of reducing sugar D-glucose from starch/min or lactose/min, under the assay conditions[14].

α- Amylase enzyme assay[15]

For the enzymatic assay, 1 ml of water sample and 1 ml of 1% soluble sterile starch solution in 2ml of 1M sterile citrate phosphate buffer of pH6.4 were added in a test tube. The reaction mixture was incubated for a time course of 5, 10, 15, 20, 25, 30 minutes in a water bath at 35°C then 2ml DNS reagent were added to stop the reaction and the tubes were kept boiling for 5 minutes for color development. The tubes were cooled under tap water then absorbance was measured at 540 nm.

β- Galactosidase enzyme assay[6]

For the enzymatic assay, 1 ml of water sample and 1 ml of 100 mM sterilized lactose solution in 2 ml of 80mM sterile potassium di-hydrogen phosphate buffer of pH7 were added in a test tube. The reaction mixture for a time course of1, 2, 5, 10, 15, 20, 25, 30 minutes in a water bath at 35° C. then 2ml DNS reagent were added to stop the reaction and the tubes were kept boiling for 5 minutes for color development. The tubes were cooled under tap water then absorbance was measured at 540 nm.

Bacterial counts

Total bacterial counts of water samples, as indicated in figure 1, were determined according to the method of Hobbie *et al.*,(1977) [16] whereas 2 ml of water samples were incubated with 2 ml 0.1 % w/v acridine orange stain for 2 minutes then the solution was filtered through 0.45 µm black stained nitrocellulose filter papers[17] using vacuum pump filtration system. The total bacterial counts were determined according to this law[18] ($N = S_1$ a n /v S_2) Whereas; N was the bacteria number, $S_1$was the area of the filter μm^2, a was the number of cells averaged of all fields, n was the index of breeding water samples ml, v was the volume of suspension that was filtered ml, S_2 was the area of the microscopic field of view μm^2.

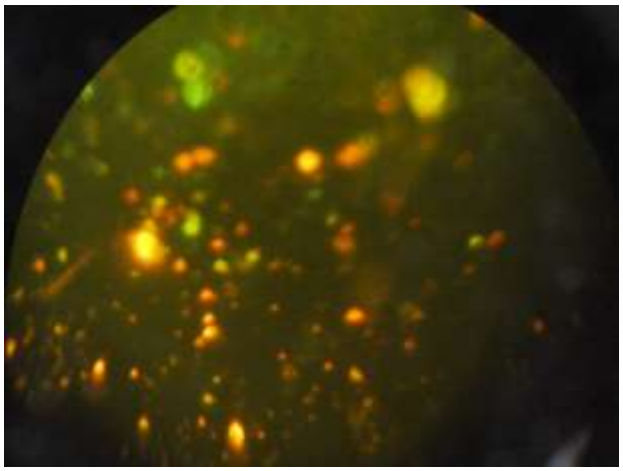

Figure 1: Epifluorescent microscopic examination of total bacterial counts of Alexandria site water samples during spring season.

Water analysis

Chemical analysis of water samples was established during the four successive seasons. Further study of the microbial enzymatic activities of α-Amylase and β-Galactosidase and determination of the environmental factors as well as the nutrient salts in the natural marine water which could induce some effects on the microbial enzymatic activities were carried out. Water analysis involved the determination of surface water temperature by submerging a graded glass mercury thermometer under surface water, chlorophyll a was spectrophotometrically determined[19] and water salinity was measured using Master Refractometer ATAGO. Cl^- concentrations were determined according to Mohr's titration method with the use of 0.1M of $AgNO_3$ solution and 5% di-potassium chromate indicator and Ca^{+2} and Mg^{+2} were determined using the titrimetric method of 0.01M EDTA- disodium salt solution in presence of Eriochrome Black T (EBT) and muroxide indicators[20]. The concentrations of SO_4^{-2} were determined according to the turbidity method which involves addition of $BaCl_2$crystals to ensure fine and stable suspension of $BaSO_4$ at a pH of about 4.8[21] and the degree of the turbidity was measured spectrophotometrically at 490 nm. The concentrations of Na^+ and K^+ were determined using the flame photometric method[22] while the trace metals of Fe^{+3}, Cu^{+2}, Mn^{+2} and Zn^{+2} were determined using the atomic absorption method[22].

Statistical analysis

SPSS 16.0 was used for the correlation of enzymatic activity results of α- Amylase and β-Galactosidase with the measured chemical and physical parameters as well as the total bacterial counts.

RESULTS AND DISCUSSION

The time courses of α- Amylase and β- Galactosidase were minimized to the three most time courses when the highest activities were recorded for both enzymes which were 5, 10 and 15 minutes.

In general, the enzymatic activity results of β- Galactosidase were higher than those of α- Amylase enzyme in water samples of both sites. The highest activities of α- Amylase and β- Galactosidase were recorded at the 5^{th} minute of the reaction start in water samples of both sites then they were gradually decreased to the lowest activity results at the 15^{th} minute with the exception that during autumn 2014 season, water samples of Port Fouad site recorded the highest activity results of α-Amylase at the 10^{th} minute while for β- Galactosidase enzyme, the activity increased gradually from the 5^{th} minute to the 15^{th} minute.

The results shown in table 1 indicated that the lowest enzymatic activity results of both α- Amylase and β- Galactosidase were mostly recorded during summer 2015 season which was confirmed by the undetectable activity (UD) activities that were recorded in Alexandria site and Port Fouad site as well. The UD activity results of both enzymes could be considered as zero results yet, it was illogically to record the enzymatic activity of an enzyme obtained from an open system, the Mediterranean Sea, as zero activity because the open system contains large communities of microorganisms whether being bacterial, fungal or other microorganisms that can degrade carbohydrates. The possible logical reasons for the UD activities of α-amylase and β-galactosidase could be attributed to the lacking of the substrates during certain seasons or time duration of the year, the variation of microbial communities during the different seasons of the year, the seasonal changes that occur in the weather and the environmental conditions, presence and absence of some nutrient salts and trace metals in the water samples during different seasons of the year and changes of the physical parameters on which the activities of different enzymes greatly depend on including pH values, temperature degrees and salinity.

Table 1: β-Galactosidase enzyme activity and α-Amylase enzyme activity water samples in (U/ml) of Alexandria site (Alex) and Port Fouad site (P.F) during four successive seasons. (UD, Un-Detectable), (m, minutes).

β-Galactosidase enzyme activity of water samples in (U/ml)									**α-Amylase enzyme activity of water samples in (U/ml)**							
m	**Autumn 2014**		**Winter 2015**		**spring 2015**		**Summer 2015**		**Autumn 2014**		**Winter 2015**		**spring 2015**		**Summer 2015**	
	Alex	P.F	Alex	P.F	Alex	P.F	Alex	P.F	Alex	P.F	Alex	P.F	Alex	P.F	Alex	P.F
5	2.6	0.12	6.05	5.2	1.3	4	UD	0.71	3.5	0.55	1.42	3.9	2.7	3	0.17	U.D
10	1.3	0.17	2.28	2.6	0.7	2.7	UD	0.32	1.8	1.3	0.73	2.2	1.55	1.11	0.18	U.D
15	0.9	0.19	1.26	1.53	0.55	1.4	UD	0.12	1.2	0.7	0.55	1.95	1.4	1.02	0.18	U.D

The statistical analysis results of α- Amylase enzyme that were recorded in the water samples of Alexandria site showed non-significantly weak correlations (r = 0.323) to those results recorded in water samples of Port Fouad site indicating presence of some variations between the enzymatic activity results of both enzymes in the water samples of both sites. The correlation results of the total bacterial counts, as indicated in table 2, and α- Amylase enzyme activity of the water samples of Alexandria site and Port Fouad site showed some variations whereas at Alexandria site, α-Amylase enzyme activity showed no correlations with the total bacterial counts while at Port Fouad site, negative correlations (r = - 0.329) were recorded between the enzymatic activity of α-Amylase enzyme and the total bacterial counts. However, similar results were recorded in case of β-galactosidase enzyme activity for both sites whereas negative correlations (r = - 0.674, r = - 0.1)

were recorded with the total bacterial counts of water samples of Port Fouad site and Alexandria site respectively.

Table 2: The total bacterial counts of water samples in ($\times 10^8$ cell/ml) of Alexandria site (Alex) and Port Fouad site (P.F) during four successive seasons.

Season /location	Autumn 2014	Winter 2015	Spring 2015	Summer 2015
Alex	0.8	0.3	0.1	0.4
P.F	7	0.1	0.1	0.6

Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Fe^{+2}, etc[23]. The activity of different β-galactosidases also depends on presence of ions. The fungal β-galactosidases are active without ions as cofactors, sometimes the yeast β-galactosidases require ions, such as[24] Mn^{+2}, Na^{+}, Mg^{+2}, K^{+} while on the contrary, Ca^{+2} and heavy metals inhibit the enzymatic activity of all β-galactosidases[25] therefore, the nutrient salts and trace metals were correlated with the activity results of α-Amylase enzyme during the seasonal duration of autumn 2014 to summer 2015 to study the effect of these nutrients on the activity of the enzyme in natural water systems. Generally, α-Amylase and β-Galactosidase enzymes showed higher activities during the cold seasons than the warm seasons at temperature ranges of (16°C - 22°C). These results were confirmed by the strong negative significant correlations ($p < 0.01$) between the water temperature degrees during the cold and warm seasons with the highest and lowest activities of α-Amylase and β-Galactosidase enzymes.

During the seasonal duration of autumn 2014 to summer 2015, it was noticed that the activities of α-Amylase and β-Galactosidase enzymes of the water samples of Alexandria site were inversely related to the increase in water salinity and chlorinity values; these results were confirmed by the strong negative correlations between the activity of α-Amylase enzyme ($r = - 0.81$, $r = - 0.97$), β-Galactosidase enzyme ($r = - 0.64$, $r = - 0.23$) and salinity and chlorinity values respectively while contrary results were recorded in case of Port Fouad water samples whereas positive correlations were recorded between the enzymatic activity values of α-Amylase enzyme ($r = 0.67$, $r = 0.62$), β-Galactosidase enzyme ($r = 0.62$, $r = 0.87$) and water salinity and chlorinity values.

The nutrient salts and trace metals were correlated with the activity results of α-Amylase enzyme during the seasonal duration of autumn 2014 to summer 2015 to study the effects of these nutrients on the activity of the enzyme in natural water systems. The statistical analysis results that were applied on the chemical results and the enzymatic activity results of α-Amylase enzyme showed non-significant correlations ($p > 0.05$) between the enzyme activity and the concentrations of Na^{+}, K^{+}, Ca^{+2}, Fe^{+3} and Zn^{+2} while on the other hand, negative correlations occurred with the

concentrations of Cu^{+2} and Mn^{+2}indicating the reversed effect of copper and manganese ions on microbial α-Amylase enzyme activity however, positive correlations occurred with the concentrations of Mg^{+2} which indicated that magnesium ions may induce an impact stimulating the microbial activity of α-Amylase enzyme. Likewise, the activity results of β-Galactosidase enzyme were correlated with the same nutrient salts where non-significant correlations ($p > 0.05$) occurred between the enzyme activity results with the concentrations of Na^{+}, K^{+}, Mg^{+2}, Cu^{+2} and Zn^{+2} however, strong negative correlations occurred with Ca^{+2}, Fe^{+3} and Mn^{+2} which indicated that high concentrations of these metal ions could induce reversed effect on the microbial enzymatic activity of β-Galactosidase enzyme.

There is no doubt that microbial enzymes play a major role in the diagnosis, treatment, biochemical investigation, and monitoring of various dreaded diseases including Amylase enzyme which is very important enzyme that has been vastly studied and has great importance in different industries and therapeutic industry[26]Therefore the microbial enzymes in marine water habitats as well as fresh water habitats need further studies to determine their advantages and uses in the different fields of life.

According to table 2, it was noticed that water samples of Port Fouad site mostly recorded higher results of the total bacterial counts than those recorded in the water samples of Alexandria site especially during the autumn season which corresponded to the enzymatic activity results of α-Amylase enzyme and β-Galactosidase enzyme recorded in the water samples of both sites during the same season while both sites recorded the lowest total bacterial counts during the spring seasons which was attributed to the highly observed algal blooms during this season at the surface water and sedimentary rocks on the shores of both Port Fouad and Alexandria sites whereas the algae-bacteria interactions are complex and can be recognized as competition, commensalism or parasitism. Microalgae may promote or inhibit bacterial growth by production of organic exudates and toxic metabolites[27, 28].Bacteria, on the other hand, may have a stimulating effect on the algae through decomposition of organic metabolites or through the production of simulative substances for algal growth[28, 29].

During the four successive seasons of the present study, it was noticed that the surface water salinity of Alexandria site was always much higher than those recorded in Port Fouad water samples whereas during autumn, winter and spring seasons, surface water salinity of Alexandria site was 45‰ and it was raised to reach 50‰ during summer 2015 season while at Port Fouad site, surface water salinity was recorded as 35‰ during the autumn season and then it was raised to reach a constant value of 40‰ during the following three successive seasons. Therefore, the

seasonal mean of salinity values recorded for Alexandria site and Port Fouad site were 46.3‰ and 38.3‰ respectively. This high variations between the surface water salinity of Alexandria site and Port Fouad site could be the major factor affecting on the variations between the enzymatic activity results of both sites which were especially observed during the autumn season when both enzymes were not following the same pattern of increasing and decreasing during the same time course in the water samples of both sites. Though the high variations of salinity values between the two studying sites, the chloride ion concentrations showed no large variations during the seasonal duration of the present study whereas the seasonal means of Cl^- ions as mentioned in table 3 were 22048.17 mg/l and 21554.44 mg/l for Alexandria site and Port Fouad sites respectively. It was also noticed that most of the measured nutrient salts and trace metals were recorded in higher concentrations in the water samples of Port Fouad site than those of Alexandria site including Mg^{+2}, Ca^{+2}, K^+, Cu^{+2} and Mn^{+2} while on the other hand, the concentrations of Na^+, Fe^{+3} and Zn^{+2} were slightly higher in the water samples of Alexandria site than those of Port Fouad site. ‘

Table 3:The mean results of the surface water salinity values (‰), concentrations of the nutrients salts and the trace metals in (mg/l) that were measured in the water samples of Alexandria site and Port Fouad site during the seasonal duration of autumn 2014 to summer 2015.

parameters	Alexandria site	Port Fouad site
Salinity	46.3	38.3
Cl^-	22048.17	21554.44
Mg^{+2}	915.4	1131.1
Ca^{+2}	223.45	268.58
Na^+	438.5	380.2
K^+	8.4	12.13
Fe^{+3}	0.65	0.502
Cu^{+2}	0.025	0.25
Zn^{+2}	0.31	0.12
Mn^{+2}	0.41	0.44

Depending on the variations of water salinity and chlorinity as well as the nutrient salt concentrations along with the concentrations of the trace metals and since the Amylases and all different β-Galactosidases are metal dependent[23, 24] it has become clear that a slight change in the concentrations of certain nutrient salt or certain trace metal along with the environmental parameters of temperature, salinity and pH, the activity of microbial enzymes in marine water systems could change drastically during the different seasons of the year even at the same studying site.

CONCLUSION

The present study provided some knowledge on the microbial enzymes in water samples of marine sources and possible factors controlling and limiting their activities. In conclusion, the microbial enzymatic activity results neither being predictable nor constant especially when measured in natural open system as in case of the Mediterranean Sea water. These results showed that although the enzymatic activities of α- Amylase enzyme and β-galactosidase enzyme were measured in marine water samples of the same source, the Mediterranean Sea, there were drastic spatial and seasonal variations which logically corresponded to the physical and chemical variations as well as the environmental variations that occur during the year. The negative correlations between the total bacterial counts and the enzymatic activity results of both enzymes in water samples of Alexandria site proved that not only the bacterial community can impact on the enzymatic activity of these enzymes but also other microbial communities including fungi and yeasts which can induce more impact depending on the nature of available substrates, microbial interactions and the suitable environmental factors for the enzymatic activity.

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