

Physiological study for extracted surface layer from *Aeromonas hydrophila* isolated from diarrhea

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Abstract:

A total of 349 stool samples were collected from the patients with diarrhea during the period from December 2014 to May 2015, the results show that there were 11(3.15%) positive isolates of *Aeromonas hydrophila*, these isolates were identified by cultural method ,biochemical tests and confirmed by API 20E and Vitek 2 system. Tryptose soy agar that contained Congo red stain was used to detect *A. hydrophila* isolates that produced surface layer protein (S-layer) ,eight (8) isolates was given positive result on this media. The S-layer protein was extracted by two methods, Sodium Dodecyl Sulphate (SDS 0.05%) solution was given significantly higher protein concentration in compared with LiCl 5M solution. Then it was precipitated by two methods also, ammonium per sulfate 80% was given significantly higher protein concentration in compared with acetone Con.95%. The molecular weight of S-layer protein was determined by SDS-PAGE and the result reveal that it was 52 KDa. The physiological factors that affecting on the production of S-layer protein from *A. hydrophila* was investigated by incubate the inoculated production media at different degree of temperature, pH, incubation period ,at the ordinary and shacking incubator, and by add (Glycine, Tyrosine ,Lysine ,Alanine) in two Con. 0.5mg/L and 1mg/L. The result show that all these factor affecting on the production of S-layer protein and the highest protein concentration was obtained at temperature 37 °c, pH 7, after incubation period 24 hrs., at shacking incubator, by add 1mg/L of glycine.

Keywords: *Aeromonas hydrophila* ,Surface layer protein, Physiological factors.

Introduction

Aeromonas spp. are Gram negative ,short rod shape ,facultative anaerobes resistance to O/129 vibrio static and non-spore forming They are generally motile by polar flagella recently assigned family Aeromonadaceae, [1,2,3]. *Aeromonas spp.* are pathogenic to fish, amphibians and also humans [4]. They are ubiquitous microorganisms found in both aquatic and environmental habitats such as estuary sediment ,sea water , sea grass, sea food, and drinking water[5,6]. *Aeromonas*

infections are typically acquired through two routes, ingestion of contaminated water or food with this bacteria, or through contact of the organisms with a break in the skin [7]. *Aeromonas hydrophila* is one species of the genus *Aeromonas* that received increasing attention as opportunistic pathogens and enterotoxigenic pathogens, because of its association with human diseases, aquatic and terrestrial animals infections [8,9]. Isolation of *A. hydrophila* from water and food sources like fish, and the increasing resistance of this organism to antibiotics and chlorination in water, presents a significant threat to public health [10]. Pathogenicity of *A. hydrophila* infection is complex and multi-factorial [11] and it's attributed to a multiple virulence factors, including cell structural: lipopolysaccharide (LPS), outer membrane proteins (OMPs), pili and flagella, type III secretion system (T3SS) acts as adhesion structures and extracellular factors such as exo-toxin, aerolysins, hemolysins, enterotoxin and siderophore that seem to play an important role in pathogenesis [12,13]. Surface array protein or S-layer are monomolecular arrays made up of a single protein species and represent the simplest biology membrane developed during evolution. S-layer composed the outer most layer of the cell envelope of prokaryotic (archaea and bacteria) [14].

A. hydrophila and *A. salmonicida* can cause disease in salmonids in freshwater and marine environments. Typical strains of both species are responsible for furunculosis, a fatal disease of these fishes, the S-layer is essential for virulence of both organisms, a crude acid-extract of an S-layer of *A. hydrophila* strain has been used for immunizing channel catfish. When immunization was performed with the S-layer protein-containing extract emulsified in Freund's incomplete adjuvant (FIA), catfish were protected against this bacteria by subsequent experimental challenge with the homologous virulent bacteria was considered a good vaccine candidate because of its surface location and its immunological properties [15,16]. S-Layer protein of *A. hydrophila* used as a vaccine in Nile tilapia (*Oreochromis niloticus*) fish against the pathogenic *A. hydrophila*, the vaccine reduced the probability of death of the vaccinated fish when compared to unvaccinated [17].

Materials and Methods

Collect of stool samples

A total of (348) stool samples were collected between December 2014 and May 2015 from patient whom suffering from diarrhea. before starting their antibiotic therapy, samples were collected from Babylon hospital for maternity and pediatrics.

Isolation and identification of *A. hydrophila*

One gram of each sample was briefly emulsified in 3ml of sterile 0.85% (w/v) saline and vortexed for 30sec. Organ debris was allowed to settle down for 5 min. the samples were put it in alkaline peptone water (pH9) and sub cultured after incubation at 37C for 6 h. onto to MacConkey agar, *Aeromonas* agar at 37C for 24hrs. [18;19]. The strains first identified as *Aeromonas* spp. According to colony morphology on MacConkey agar, Blood agar, Thio sulphate citrate bile salt sucrose agar (TCBS) agar and *Aeromonas* agar (AIA), and by microscopic morphology (Gram stain) and by chemical tests (Oxidase, Catalase, Indole, Methyl red, Voges-Proskauer, Citrate utilization, Gelatin liquefaction, kligler iron and Urease test) and by String test, Lysine decarboxylase, Arginine decarboxylase, Ornithine decarboxylation for differentiated from *Vibrio cholera* and the diagnostic of these strains confirmed by Api 20E and Vitic 2 system (Biomérieux, France).

Detection the presence of S-layer in *A.hydrophila* isolates

This test was performed by inoculating tryptose soy agar that contained Congo red stain with *A. hydrophila* isolates by streaking method, and then it was incubated at 37°C for 24 hours[20,21].

Extraction of S-layer protein

In this study S-layer protein was extracted from *A. hydrophila* isolates by two methods, and then it was compared between methods according to protein concentration that was measured by Bradford method [17,22].

First method: extracted of S-layer protein by SDS 0.05%

In this method S-layer protein was extracted according to the modified method of [23,24,25].*A.hydrophila* was reactivated, then 1ml of bacterial suspension was added to tryptose soy broth and incubated at 37°C for 24hrs. The bacterial cells was collected by centrifuge at 6000 rpm for 10 min. The pellet of cells was suspended by phosphate buffer solution pH7 and washed 3 times by centrifuge at 6000 rpm for 15 min. Then the pellet was suspended by 5 ml SDS 0.05% solution and incubated for 10 min at 37°C. Then the bacterial suspension was centrifuged at 12000 rpm for 15 min at 4°C, the supernatant represent the crude extraction(CE) was used and kept in refrigerator at 4°C.

Second method: extracted of S-layer protein by lithium chloride 5M

In this method S-layer protein was extracted according to the modified method of [26]. The same steps of the first method was performed but in the second method the cell pellet was suspended by lithium chloride 5M after washed the bacterial cells, then the bacterial suspension was incubated at shaking incubator at 200 rpm for 1 hr. at 37°C. after that it was centrifuged at 12000 rpm for 15 min at 4°C, the supernatant was used and kept in refrigerator at 4°C.

Precipitation of S-layer protein

In this study two methods has been used to precipitate S-layer protein from crud extraction (CE) and it has been compared between methods. the S-layer protein Con. was measured by Bradford method [17,22].

First method: Precipitation by ammonium per sulfate with Saturation ratio 80%

This method was done according to method of [27] Ammonium per sulfate (51.6 g) was gradually added to (100 mL) of crud extraction (CE) of S-layer protein with continuous mixing by magnetic stirrer at 150 rpm for 10 min at 4°C. The solution was centrifuged at 10000 rpm for 15 min. the supernatant was removed and the sediment (S-layer protein) was used. Phosphate buffer saline (5ml) was added to the sediment (S-layer protein), then it was kept in refrigerator at 4°C.

Second method: Precipitation by absolute acetone with Con.95%

This method was performed according to [28] Acetone (95%) was cooled to -20°C. The crud extract (CE) was putted into sterile test tube. Cooled acetone (95%) was added to the crud extract (CE) in percentage (2:1), 2 ml of Cooled acetone (95%) to 2 ml of the crud extract (CE). The solution was centrifuged at 10000 rpm for 15 min at 4°C, the supernatant was removed and the sediment (S-layer protein) was used. Phosphate buffer saline (5ml) was added to the sediment (S-layer protein), then it was kept in refrigerator at 4°C.

SDS- Poly acrylamide gel electrophoresis (SDS-PAGE) of S-layer protein

This assay was performed according to the method[29]to determine the molecular weight of S-layer protein. The proteins were visualized in 15% polyacrylamide gel with denaturing conditions (SDS-PAGE) .After preparation of the gel, the protein extracts 50 µL were added to a mix containing 50µL of sample buffer and heated in a water bath at 100°C for 5 min before being applied in the gels. After electrophoresis, the gels were stained with Coomassie Blue [30] and the protein standards were registered in an image-capturing system.

Effect of the physiological factors on the production of S-layer protein

Three isolates of *A. hydrophila* bacteria (second , fifth, and eighth isolates) has been chosen to study the effect of five physiological factors on the production of S-layer protein. In this experiment S-layer protein has been extracted by first method (0.05% SDS solution) [23,24,25] and it was also precipitated by the first method (Ammonium sulfate 80%) [27].

The physiological factors were investigated by inoculate the production media(tryptose soy broth) with *A. hydrophila* isolate and incubated at different degree of temperature (5, 10, 15, 20, 25, 30, 37, 40, 45 and 50)°c, pH(1,4,7,10), incubation period (4,8,12,16,20,24,28 and 30) hrs., at the ordinary and shacking incubator, and by add four type of amino acid (Glycine, Tyrosine ,Lysine ,Alanine) in two Con. 0.5mg/L and 1mg/L.

Statically analysis

Data were processed and analyzed by using Anova one way, and the Least Significant Difference (LSD) to determine the significant difference between the different factor in the tests by using the using statistical program SPSS 19 and the results were expressed as(Mean±S.D).P-Values below 0.05 were considered to be statistically significant.

Results and Discussion

Isolation of *A. hydrophila*

Out of three hundred forty nine (349) stool samples analyzed,11(3.15%) samples were positive for *A. hydrophila* bacteria as show in the Table1. This result is similar to the result that obtained from the local study[21]that reported 2.7% (13 isolates) of *A. hydrophila* from 479 stool samples in Babylon province .

Table 1. Percentage of *A. hydrophila* isolates from stool samples.

Bacterial type	NO. of samples	Percentage
<i>A. hydrophila</i>	11	3.15%
Other type of bacteria	338	96.84%
Total	349	100%

However ,the result of this study revealed that *A. hydrophila* infected children from 1-6 years old more than other ages in this study and having 3.84%(6 isolates) from 156 stool samples as show in the Table 2., this result is similar to the

finding of the local study [31] that reported 4.08% (12 isolates) from 294 stool samples from 1 month to 6 years old in AL-Samawa province.

Table 2. Percentage of *A. hydrophila* isolates according to the age group.

Categories of patient	NO. of stool samples	NO. <i>A. hydrophila</i> isolates	Percentage
1-6 years	156	6	3.84%
7-12 years	109	3	2.75%
13-18 years	84	2	2.38%
Total	349	11	3.15%

Identification of *A. hydrophila*

The colonies of *A. hydrophila* that was grown on culture media appeared 1-3mm in diameter as with [32] The positive isolates for *A. hydrophila* revealed pale, non-lactose fermenters, small colonies on the MacConkey agar because it is unable to ferment lactose sugar and it was showed smooth, rounded, convex, β -hemolysis colonies on blood agar and yellow-shine color on TCBS agar due to their ability to ferment sucrose sugar, the results agreed with other studies [13,33] *Aeromonas* agar with ampicillin supplement was also used, it was appeared in dark green color, opaque with dark center this is agree with [3]. The microscopically examination was exhibited Gram negative rod bacteria, that was appeared singly, in pairs, or even as short chains on glass slide this agreed with [32]. The biochemical tests used to confirmed the initial diagnosis of *A. hydrophila* as show Table 3. the positive isolates of *A. hydrophila* were given apposite result of each oxidase, catalase, indole, methyl red, vogues –proskauer, citrate utilization and gelatin liquefaction tests. It had the ability to ferment glucose on kligler iron agar (Alk/Acid). In the top of the slant agar, the red color (Alkaline) was appeared while in the bottom of the slant agar, the yellow color (acidic) was appeared with no H₂S production. It was given a negative result for urease test, this is agreed with [34].

Table 3. Biochemical tests of *A. hydrophila* isolates

No.	Type of test	Result
1	oxidase test	+
2	Catalase test	+
3	Indole test	+
4	Methyl red test	+

5	Vogues –Proskauer test	+
6	Citrate Utilization test	+
7	gelatin liquefaction test	+
8	kligler iron agar test	Alk/Acid
9	Urease test	-

String test was done to distinguished *A. hydrophila* which was negative to this test from *V. cholera* which was positive to this test. amino acid utilization test was also performed, In this test *A. hydrophila* was given a positive result to lysine decarboxylase and arginine decarboxylase and a negative result to ornithine decarboxylase, this result was agreed with [35] as show in the Table 4. API 20E and Vitek 2 System was also used to confirm the identification of *A. hydrophila* isolates.

Table 4. Differentiate between *A. hydrophila* and *V. cholera* via string test and amino acid utilization test

No.	Type of test	<i>A. hydrophila</i>	<i>V. cholera</i>
1	String test	-	+
2	Lysine decarboxylase	+	+
3	Arginine decarboxylase	+	-
4	Ornithine decarboxylase	-	+

Detection the presence of S-layers in *A. hydrophila* isolates

Out of eleven(11) isolates of *A. hydrophila*, eight(8)isolates was revealed a positive result in this test. the isolates that produce S-layers protein was given a dark red colonies or the same color of the medium as in the Figure 1 ,while the isolates that was not produce S-layers was given colorless or light orange colonies, This is agree with other study that also used Congo red agar (CRA) as a differential media for the fish pathogen *A. salmonicida* and it had improved that the S-layers protein absorb Congo red stain therefor the isolates that have S-layers appear in dark red colonies on the medium [20,36].

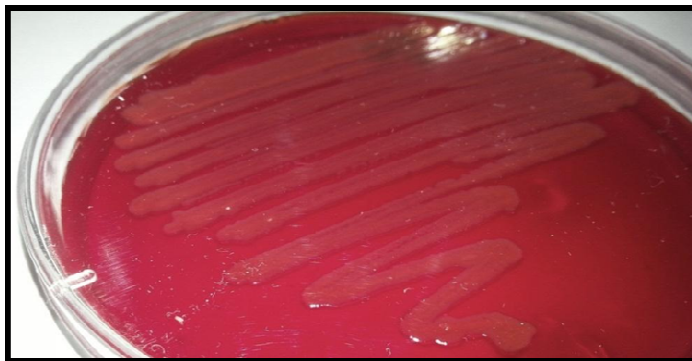


Fig.1: S-layers in *A. hydrophila* appeared as dark red isolates on tryptose soy agar that contains Congo red

Extraction of S-layer protein from *A. hydrophila*

The first method (SDS 0.05%) was given high protein concentration for all *A. hydrophila* isolates with mean value $769.07 \pm 41.43 \mu\text{g/ml}$ in compare with the second method (lithium chloride, 5M) that was given protein concentration with mean value $498.53 \pm 40.48 \mu\text{g/ml}$. Based on determined protein concentration and statically analysis there's a significant difference at ($P < 0.05$) between these two methods. Isolates of *A. hydrophila* was produce different amount of protein concentration ,the fifth (5) isolate was given the highest protein concentration, it was given $828 \mu\text{g/ml}$ and $549.42 \mu\text{g/ml}$. while the seventh(7) isolate was given the lowest protein concentration ,it was given $713.71 \mu\text{g/ml}$ and $442.28 \mu\text{g/ml}$. in The first method (SDS 0.05%) and the second method (lithium chloride, 5M) respectively as show in the Figure 2.

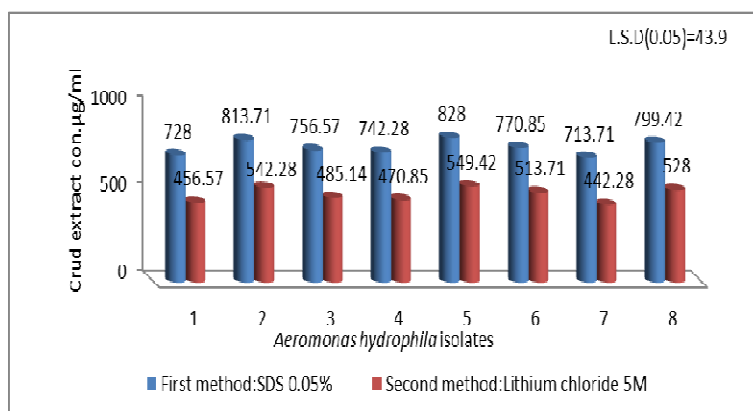


Fig.2: Concentration of crud extract for S-Layer protein of *A. hydrophila* isolates in two methods

The S-layer protein in *A. hydrophila* bacteria is composed of protein subunits that are bound to the underlying cell wall through the hydrogen bonds that are important in stabilizing the layer with other cell wall components , S-layer protein can be extracted from the cell surface by different chemicals at a concentration capable of disrupting H-bonds [37]. Both of Sodium Dodecyl Sulphate (SDS 0.05%) solution and lithium chloride (LiCl, 5M) solution that used in our study considered as a hydrogen –bond breaking agent [24,38]that break the hydrogen bonds and affecting the stability of S-layer protein by weakening the hydrophobic interaction.

Precipitation of S-Layer protein

The first method(ammonium per sulfate 80%) was given a high protein concentration for all *A. hydrophila* isolates with mean value $625.5 \pm 39.83 \mu\text{g/ml}$ in compare with the second method (acetone 95%) that was given protein concentration with mean value $371.23 \pm 58.93 \mu\text{g/ml}$. Based on determined protein concentration and statically analysis there's a significant difference at ($P < 0.05$) between these two methods. Isolates of *A. hydrophila* was different in their production of s-layer proteins .The second ,fifth and eighth isolates was given a high level of protein concentration in compare with other isolates ,in the first method(ammonium per sulfate (0.05%)) it was given 670.85 , 678 and 656.57 $\mu\text{g/ml}$ respectively. hile in the second method(acetone 95%) it was given 428.1, 435.14 and 413.72 $\mu\text{g/ml}$ respectively as show in the Figure 3.

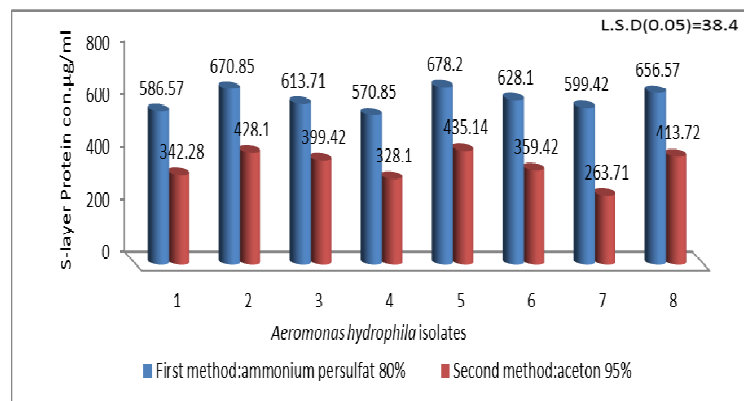


Fig.3:S-layer protein concentration of *A. hydrophila* isolates in two methods.

According to the result that the S-layer protein concentration that obtained by the first method were higher than the second method, therefore ammonium per sulfate (0.05%) was preferred in the precipitation of S-layer protein in this study. In addition other study precipitated S-layer protein from the crude extraction by ammonium per sulfate [17].

SDS-Polyacrylamide Gel Electrophoresis of S-layer protein

The result revealed that the molecular weight of S-layer protein was 52KD as show in the Figure 4 .It was detected in comparison with the molecular weight of standard proteins in rang 10-180KD ,this result agree with the finding of other studies that reported the molecular weight of S-layer protein *A. hydrophila* was 52KD which also determined by SDS-PAGE technique [17,39].

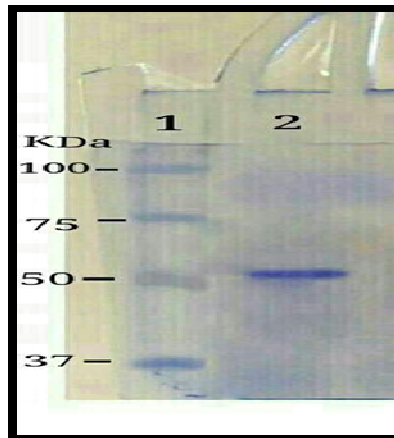


Fig.4:SDS- PAGE gel(15%poly acrylamide)analysis of S-layer protein Lane 1:lader protein ,Lane 2:sample.

Effect of the physiological factors on the production of S-layer protein

Temperature

The result revealed as show in the Figure 5 that the highest S-layer protein concentration was obtained at 37°C with mean value $679.42 \pm 3.79 \mu\text{g/ml}$. While the lowest protein S-layer concentration was obtained at 45°C with mean value $52.76 \pm 3.57 \mu\text{g/ml}$. Statically analysis show that the incubation temperature has significantly effect on the production of S-layer protein. The production of S-layer protein has a direct relationship with the growth of bacteria that greatly affected by the temperature degree of incubation. *A. hydrophila* isolates that considered as a human pathogen and caused diarrhea illness in human classified as a mesophilic bacteria [13].

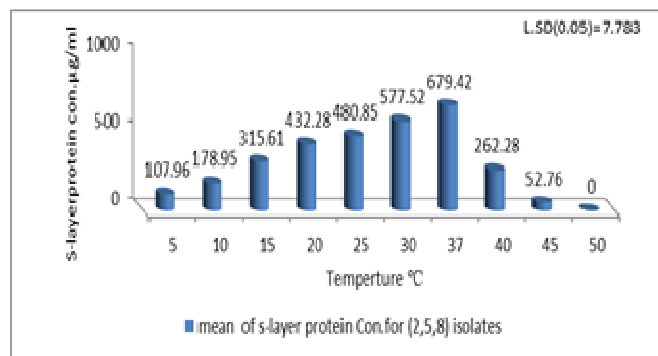


Fig.5: Effect of incubation temperature on the production of S-layer protein by *A. hydrophila* isolates.

The optimum rang of temperatures for the growth of *A. hydrophila* was (20-37)°C [40,41] this rang of temperature was appropriate for the enzymatic and metabolic activities of mesophilic bacteria and any increase or decrease from the optimum temperatures will effected on the growth of bacteria. Other study mention that the isolates was able to grow well at 37 °C [42], as this result revealed that the optimum rang for the production of S-layer was rang between (20-37)°C and the highest S-layer protein concentration was obtained at 37°C. But the lowest S-layer protein concentration was obtained at 45°C, and there was no significant production at 50°C, because the *A. hydrophila* isolates was not able to grow at 50°C and the thermal point of *A. hydrophila* isolates from 40 to 50 °C [42]. Other study mention that the high temperature cause loss of virulence factor including S-layer protein[20].

pH Value

The result revealed as show in the Figure 6 that the highest protein concentration was obtained at pH7 with mean value $682.76 \pm 2.19 \mu\text{g/ml}$. while pH 10 with mean value $589.90 \pm 3.62 \mu\text{g/ml}$, while the lowest protein Con. was obtained at pH4 with mean value $307.04 \pm 2.19 \mu\text{g/ml}$. and there was no significant production at pH1.

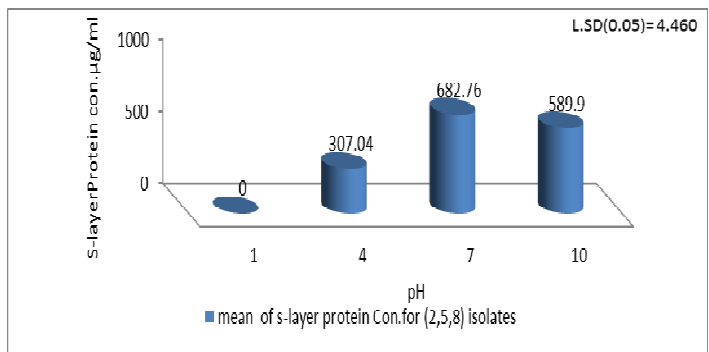


Fig.6:Effect of pH level of production media on the production of S-layer protein by *A. hydrophila* isolates.

The maximum growth of *A. hydrophila* occurred between pH 5.2 and 9.8 [43], and all *A. hydrophila* isolates show more or less similar growth at pH 7, 8, 9, while the log number of cells at pH 5, 6, 10 was found lesser than pH 7, 8, 9 [44] in the present study that the highest S-layer protein Con. was obtained at pH7 followed by S-layer protein Con. at pH 10. On the other hand the least level of pH for the growth of *A. hydrophila* isolates at the pH 4.5 [45]. Therefore the lowest S-layer protein Con. was obtained at pH 4, and there is no significant production of at pH1.

Incubation period

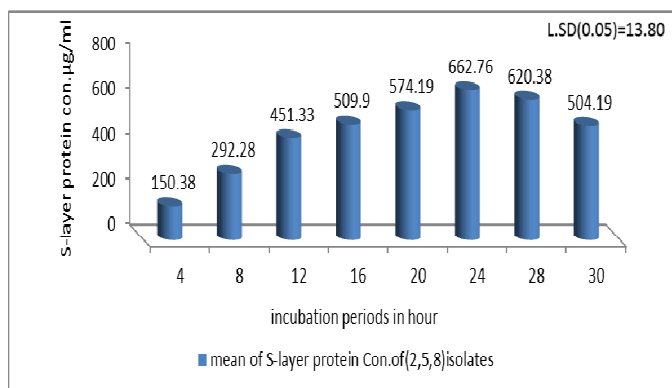


Fig.7:Effect of incubation periods on the production of S-layer protein by *A. hydrophila* isolates.

Other study mention that the bacteria produce the S-layer protein in the mid of the log phase and the end of the log phase, and the concentration of S-layer protein in the mid of the log phase was lower than in the end of the log phase [46]. In addition that there was some studies extracted the S-layer protein from *A. hydrophila* at different incubation period at 18 hrs. [39] and at 24 hrs. [47] and at 48hrs [17].

Aeration

The result revealed as show in the Figure 8 that the S-layer protein concentration with mean value $863.71 \pm 7.14 \mu\text{g/ml}$ that obtained after incubation at shacking incubator was higher than S-layer protein concentration with mean value $656.57 \pm 14.28 \mu\text{g/ml}$ after incubation at ordinary incubator. *A. hydrophila* are gram-negative, facultative anaerobic [1]. That grow under aerobic and un aerobic condition ,other study recorded that the maximal cell yield of *A. hydrophila* bacteria and it is enzymatic activities increased under aerobic condition after incubation the production media at shaking incubator [48].

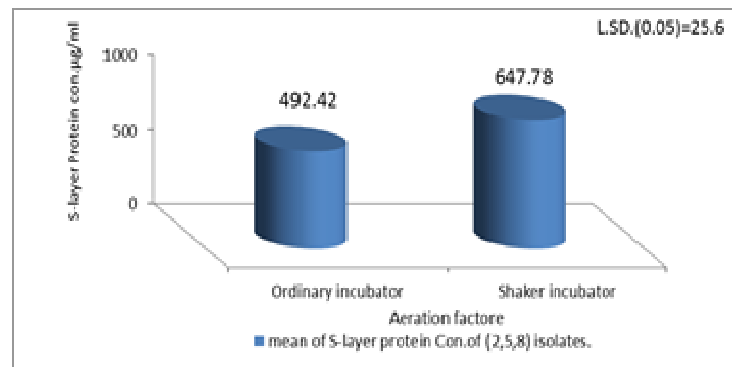


Fig.8:Effect of Aeration on the production of S-layer protein by *A. hydrophila* isolates.

The component of the production media

In the present study four type of amino acid (Glycine, Tyrosine ,Lysine ,Alanine) was added to the production media as a nitrogen source in two concentration 0.5mg/L and 1mg/L, in two experiments. In the first experiment, 0.5mg/L of the amino acid was added, the result revealed that the high protein concentration was obtained after adding glycine with mean value $1158.95 \pm 10.90 \mu\text{g/ml}$ flowed by(Tyrosine ,Lysine ,Alanine) with mean value (1138.95 ± 12.48 , 931.33 ± 7.05 , 700.71 ± 9.94) $\mu\text{g/ml}$, as show in the Figure 9.

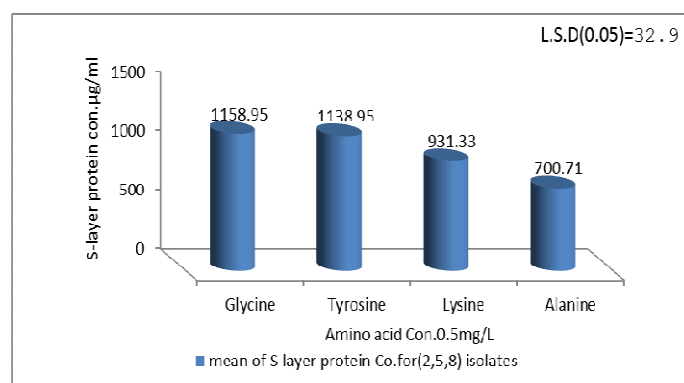


Fig.9:Effect of different amino acid with Con.0.5mg/L on the production of S-layer protein by *A. hydrophila* isolates.

At the second experiment, 1mg/L of the amino acid was added, the result revealed that the high protein Con. Was also obtained after adding glycine with mean value $1318 \pm 8.72 \mu\text{g/ml}$ flowed by(Tyrosine ,Lysine ,Alanine) with mean value (1229.90 ± 4.59 , 1100.85 ± 5.14 , 774.19 ± 9.73) $\mu\text{g/ml}$, as show in the Figure 10.

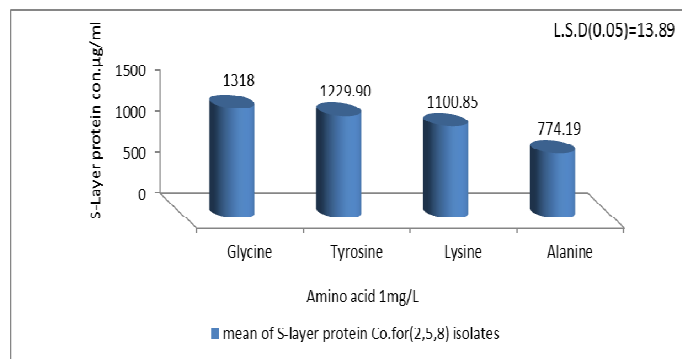


Fig.10: Effect of different amino acid with Con.0.5mg/L on the production of S-layer protein by *A. hydrophila* isolates.

In the two experiments glycine was given the highest protein concentration followed by Tyrosine, Lysine, Alanine. and the concentration of S-layer protein was increased after increasing the concentration of amino acid from 0.5mg/L to 1mg/L. Amino acid as a nitrogen source caused an increase in the growth of bacteria in the production media and the result showed a significant increase in the concentration of S-layer protein after adding amino acid. The result also showed that the S-layer protein concentration was different according to the type and concentration of amino acid, and the type of amino acid has a significant effect on the production of S-layer protein, because the nitrogen source has a different effect on the growth stimulation of *A. hydrophila* and so their production [48]. Schar-Zammaretti [49] suggested that S-layer protein is preferentially expressed under different fermentation media. Furthermore, it has been shown that the S-layer production is changed with the change in medium (such as bile salt, penicillin G)[50].

Conclusions

- (1) *A. hydrophila* bacteria are one of the causative agents of diarrhea.
- (2) Extraction of Surface layer (S-layer) protein from *A. hydrophila* by Sodium Dodecyl Sulphate (SDS 0.05%) solution and precipitation by ammonium persulfate with a saturation ratio of 80% was given a high concentration of S-layer protein.
- (3) The S-layer protein profile by SDS-PAGE analysis shows that the molecular weight of S-layer protein was 52KDa.
- (4) The physiological factors like Temperature, pH, incubation period, aeration and the components of the production media affect the production of S-layer protein by *A. hydrophila*.

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