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# Optimization of conditions for L- asparaginase production by certain bacterial strains isolated from soil at Fayoum Governorate

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**ABSTRACT:**

The cultures namely *Brevundimonas olei* No.(15),*Bacillus subtilis* No.(28) and *Bacillus cereus* No.(32) which were found to be the most producing of L-aspaginase producing isolates from soil at Fayoum Governorate, has been used in this investigation. Results showed that, the highest enzyme activity was obtained at pH 8.0 for *Brevundimonas olei* No.15 at incubation temperature 30 $^0$C. Low level of L- asparaginase production by *Bacillus subtilis* No.28 occur at pH 7.0 at incubation temperature 30$^0$C, reached its maximum level at pH 9.0 at incubation temperature 40$^0$C. Also, the effects of different carbon sources on enzyme production were studied i.e. dextrose, sucrose, Fructose, Lactose and Mannitol at the concentration of (0.5%) at various incubation period 24, 48 and 72 hrs. Results that, indicated that, *Brevundimonas olei* No.15 gave the highest enzyme yield being (444.8 IU/ml.) after 24 hrs. fermentation period using mannitol as a sole carbon source followed by dextrose (391.0 IU/ml.), lactose (390.0 IU/ml.) , sucrose (386.2 IU/ml.) and fructose (379.3 IU/ml.) respectively. Orange and potato peels were examined as a carbon source for production of L-asparaginase. Therefore, orange or potato peels were added to the modified M9 broth medium and substituted glucose. Generally results showed that, potato peels gave the highest L-asparaginase yield than orange peels for all investigated bacterial strains.

**Key words:** L- Asparaginase, Environmental conditions, Bacteria, Agricultural wastes

## 1. INTRODUCTION:

L-asparaginase (EC.3.5.1.1.) also known as L-asparaginase aminochdrolase , specially catalysis the breakdown of amino acid asparagine to aspartic acid and ammonia. It reduces the level of acrylamide that is produced during baking of starchy foods. L-sparaginase is widely distributed in plants, animals, as well as microorganisms (bacteria, yeast and fungi). In food industries, acrylamide synthesized during the process the baking and frying at high temperatures under low moisture content. As acrylamide is a potential carcinogenic. Asparagine acts as precursor in the process of acrylamide reduced.

Environmental conditions i.e. pH of the medium, incubation temperature, incubation period as well as the nutrition of the microorganisms have an effective influence on enzyme synthesis (**Arends, 1973, Pal . 1978, El-Gamel & El-Sheikh 1980 and Abou- Hamed 1996) .**

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The microorganisms can utilize a wide variety of organic matter as a source of carbon and nitrogen. This capability makes it possible to benefit from cheap agricultural or industrial organic wastes in producing enzyme. Therefore, this paper is an attempt to throw some light on some factors affecting L-asparaginase production such as pH of the medium as well as, temperature, incubation period, and different carbon sources of the fermentation medium. The cheap locally agricultural or industrial organic wastes i.e. orange and potato peels cause a serious problem to raise the recent environmental pollution. Therefore, this work aimed to find suitable and simple method for the bioconversion of these wastes for the production of L-asparaginase.

## 2. MATERIAL AND METHODS:

### 1. Microorganisms used:

The cultures namely *Brevundimonas olei* No.(15),*Bacillus subtilis* No.(28 ) and *Bacillus cereus* No.(32) which were found potent to be the most L-aspaginase isolates from soil at Fayoum Governorate, has been used in this investigation. It was identified according to morphological characteristics by Senath **et al., 1986).** Identification was further confirmed by sequencing of the 16S rRNA.

### 2- Agricultural wastes used:

Orange and potato peels in this study were obtained from local marked at Fayoum Governorate. They were air dried, crushed and then ground in a hammer mill grinding machine before use. Chemical analysis of two tested wastes carried according to **Romelle et al. (2016)** and **Joshi et al. (2020).** Their composition was as follows in Table (4).

**Table 4. some chemical composition of the tested agriculture wastes.**

| Composition*(%) | Orange peel | Potato peel |
|---|---|---|
| Protein | 9.73 | 1.20 |
| Carbohydrates | 53.27 | 12.4 |
| Moisture | 86.0 | 83.30 |
| Ash | 5.17 | 7.70 |
| Ca | 0.05 | 0.03 |
| K | 0.02 | 4.13 |

***on dry weight basis**

### 1. Fermentation experiments:

**Preparation of inoculum:**

After an incubation period of 24 hours, cultures of the selected strains obtained on modified M9 agar slants were suspended in sterilized distilled water. A 1.0 ml of culture was aseptically transformed to 250 ml Erlenmeyer flasks, each containing 100 ml. sterile inoculation medium. Flasks were incubated on a rotary shaker at 120 rpm and $37^0$C for 24 hrs.

**Fermentation:**

Ten ml of the inoculum were aseptically inoculated in 250 ml. Erlenmeyer flasks containing 100 ml. of the modified M9 growth medium and incubated on a rotary shaker of 120 rpm for 24 hr. at $37^0$C. At the end of the experiment, the content of each flask was centrifuged at 4000 rpm for 10 min. to obtain a clear liquor, containing the enzyme, followed by the estimation of enzyme activity.

**2. Factors affecting on the production of L-asparaginase by tested bacterial strains:**

In order to study the effect of different factors on L-asparaginase production. Fermentation medium of was modified to the suitable for studying the respective factors as follows:

**Effect of pH and incubation temperature:**

The pH of the fermentation medium was adjusted at 7.0, 8.0 and 9.0 at various incubation temperatures (30, 40 and 50$^0$C). At the end of the fermentation period the enzymatic activity was estimated.

**Effect of different carbon sources at various fermentation periods:**

The effect of different carbon sources on enzyme production were studied i.e. dextrose, sucrose, Fructose, Lactose and Mannitol at the concentration of (0.5%) at various incubation period 24, 48 and 72 hrs.

**Agricultural wastes used for production of L-asparaginase:**

Due to the fact, any waste product containing carbohydrates in concentration of 3% or more can be fermented, **Abd El-Bakey, (1974).** Several agricultural waste can serve as raw materials for the L-asparaginase synthesis of bacteria, **Sanghvi, *et al* (2016), Ruma, *et al.* (2017).** Orange and potato peels were selected as a carbon source for production of L-asparaginase. Therefore, orange or potato peels were added to the modified M9 broth medium and substituted glucose

**3.2.7. Media used:**

**3.2.7.1. Modified M9 agar medium (Gulati, *et al. (*1997)**

| **Composition** | g/L | |
|---|---|---|
| $Na_2HPO_4.2H_2O$ | 6.0 | |
| $KH_2PO_4$ | 3.0 | . |
| NaCl | 0.5 | |
| L-asparagine | 5.0 | |
| MgSO4.7H2O | 0.5 | |
| CaCl2.2H2O | 0.15 | |
| Glucose | 2.0 | . |
| Agar | 15.0 | |
| Distilled water | 1000 ml. | |
| Bromothymol blue | 0.017 | pH 7.0 |

**3.2.7.2. Inoculation medium: - Nutrient broth (Difico, 1985)**

| **Composition** | g/L |
|---|---|
| Beef extract | 3.0 |
| Peptone | 5.0 |
| Distilled water | 1000 ml. |
| pH 7.0 | |

**3.2.7.2. Fermentation medium Modified M9 broth medium(Gulati, *et al. (*1997)**

| **Composition** | g/L | |
|---|---|---|
| $Na_2HPO_4.2H_2O$ | 6.0 | |
| $KH_2PO_4$ | 3.0 | |
| NaCl | 0.5 | |
| MgSO4.7H2O | 0.5 | |
| CaCl2.2H2O | 0.15 | |
| L-asparagine | 5.0 | |
| Disliked water | 1000 ml. | pH 7.0 |

**L-asparaginase activity assay:**

L-asparaginase activity was determined according to **Devi** and **Ramanjaneyulu, (2016**). Quantitative detection was carried out by a Nesslerization method A 0.5 ml of the sample add to 1.0 ml of 0.1 M tris HCl buffer (a pH 8.5) and 0.5 ml of 0.04M L-asparagine solution were mixed and incubated at 37ºC for 10 min. The reaction was then stopped by the addition of 0.5 ml of 15% trichloroacetic acid. The precipitated protein was removed by centrifugation and the liberated ammonia was determined by direct nesslerization. After 2 min. 0.5 ml of nessler's reagent was added and mixed. Suitable blanks of substrate and enzyme containing sample were included in all assays. After 5 min. from the addition of Nessler's reagent, the optical density of the sample was read at 436 nm was recorded.

## Calculation:

$$\text{Units/ml} = \frac{\text{Micromoles of ammonia released}}{10 \text{ minutes} \times \text{ml enzyme in reaction}}$$

One L-aspraginase unit (IU) was defined as the amount of supernatant (enzyme) which liberate 1U mole of ammonia (ammonium sulphate as slandered curve) per minute under the optimal assay conditions.

## 3. RESULTS AND DISCUSSION:

**Factors affecting L-sparaginase production by tested bacterial strains:**

The success of most industrial fermentation depends on the inability of the microorganism to maintain balanced and economical metabolism under the prevailing conditions. The constituents of the medium and the other environmental conditions may have a marked effect on the enzyme synthesis (**Lilly 1967** and **Deman, 1981**). Therefore, the effect of the media with various constituents were studied to evaluate various carbon sources and to select the best source for the highest possible yield of L-asparaginase. In addition experiments were carried out to define the optimum environmental conditions for the maximum production of L-asparaginase i.e. pH values, incubation temperature as well as fermentation period from the three tested bacterial strains.

**Effect of the pH values at different incubation temperatures:**

The initial pH of the medium as well as incubation temperature in which the organism is grown has a great influence on enzyme production**.** Therefore an experiment was designed to investigate the effect of different pH values (7.0. 8.0 and 9.0) at various incubation temperature (30. 40 and 50 $^0$C) on L-asparaginase production by three tested bacterial strains using modified M9 broth medium.

The obtained results given in Table (1) and graphically illustrated by Figs. (1, 2 and 3) show that, the highest enzyme activity was obtained at pH 8.0 for *Brevundimonas olei* No.15 at incubation temperature 30 $^0$C. Low level of L- asparaginase production by *Bacillus subtilis* No.28 occur at pH 7.0 at incubation temperature 30$^0$C, reached its maximum level at pH 9.0 at incubztion temperature 40$^0$C.

With respect to *Bacillus cereus* No. 32, the data presented in Table (1). as well as Fig. (2) indicate that, gave the same amount of enzyme activity at pH 8.0 and 9.0 when incubated temperature at 40$^0$C. Also, data presented in Table (1) as well as Fig. (2) showed that, *Bacillus subtilis* No. (28) gave the highest enzyme activity was observed at pH 9.0 when incubated at 40$^0$C..These results come to support finding

of (**Mahajan *et al*, 2012, Singh** and **Srivasrtava, 2014, Joshi** and **Kulkarni, 2016** and **Abdelrazek *et al.*, 2019).**

**Table 1 . Effect of pH and temperature on the production of L asparaginase**

| Incubation temperatures ($^0$C) | pH values | L-asparaginase activity (IU/ml.) | | |
|---|---|---|---|---|
| | | ***Brevundimonas olei*** **No.`15** | ***Bacillus subtilis*** **No.28** | ***Bacillus cereus*** **No.32** |
| **30** | **7.0** | 152.34 | 172.29 | 168.68 |
| | **8.0** | 364.38 | 361.32 | 342.34 |
| | **9.0** | 361.32 | 359.48 | 355.40 |
| **40** | **7.0** | 249.72 | 402.73 | 418.65 |
| | **8.0** | 324.39 | 372.13 | 428.03 |
| | **9.0** | 321.52 | 418.24 | 428.03 |
| **50** | **7.0** | 172.60 | 369.27 | 423.14 |
| | **8.0** | 207.89 | 329.34 | 423.95 |
| | **9.0** | 225.64 | 364.38 | 425.99 |

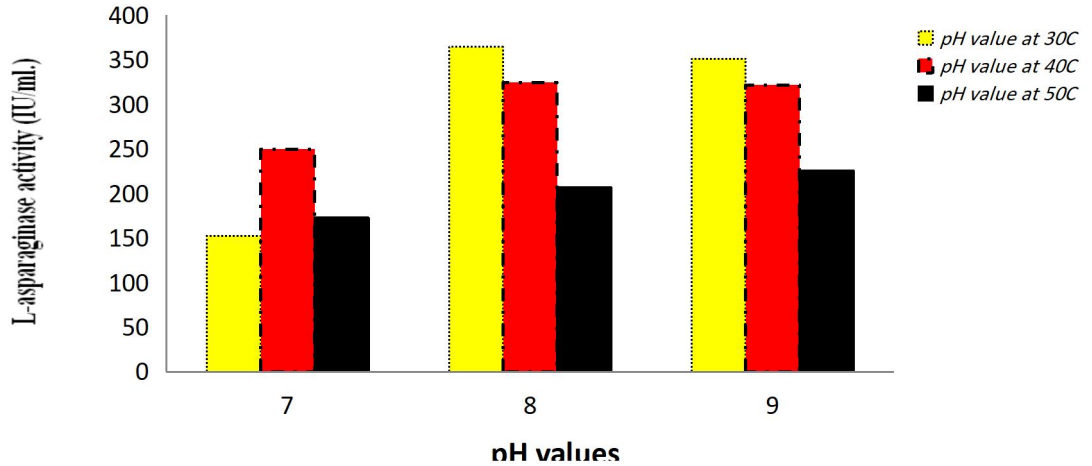


**Fig. 1. Effect of pH values at different incubation temperature on the production of L-aparaginase *Brevundimonas olei* No.15**

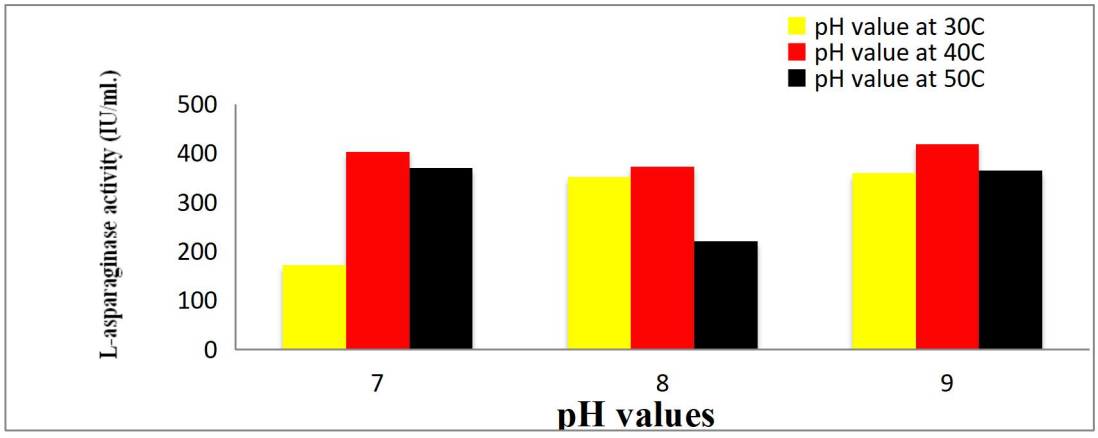

**Fig. 2. Effect of varios pH values at different incubation temperature on the production of L-aparaginase by *Bacillus subtilis* No.28**

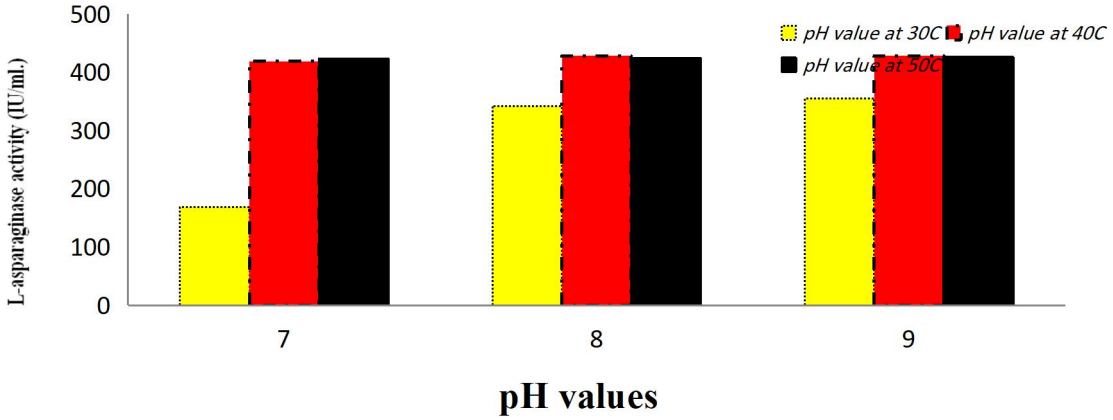


**Fig. 3. Effect of varios pH values at different incubation temperature on the production of L-aparaginase by Bacillus *cereus* No.32**

**Effect of different carbon sources at different fermentation periods:**

The experiments were designed to find the best carbon source at various fermentation periods which produce the highest amount of L-aspraginase by *Brevundimonas olei* No.15, *Bacillus subtilis* No.28 and *Bacillus cereus* No.32. Different carbon sources namely, dextrose, sucrose, fructose, lactose and mannitol were individually tested as the sole source of carbon in the fermentation medium. The fermentation period for the maximum production of L-asparaginse is one of the limiting factor for the economic success of the commercial production. **Fogarty** and **Kelly (1980)** reported that the selected organism must produce a good yield of the enzyme in a relatively short time and ideally in submerged culture. In our experiments, the fermentation period was prolonged for 72 hrs. and the enzyme activity was estimated at 24 hrs. intervals.

Data presented in Table (2) and graphically illustrated by Fig. (4) clearly indicate that, mannitol as a sole carbon source gave the highest enzyme yield being (444.8 IU/ml.) after 24 hrs. fermentation period followed by dextrose (391.0 IU/ml.), lactose (390.1 IU/ml.) , sucrose (386.2 IU/ml.) and fructose (379.3 IU/ml.) respectively for *Brevundimonas olei* No.15. Also, results revealed tthat, addition sucrose instead of glucose (basal medium) produced lower enzyme yield being (72.3 IU/ml.) than glucose and other carbon sources after 72 fermentation period.

Generally, results revealed that, increaing the fermentation period over 24 hours , the enzymatic activity decreased. The decrease in enzymatic activity with prolongation of fermentation period may be due to the repression effect of the products from the enzymatic hydrolysis of asparagine.

**Table 2. Effect of carbon sources, on asparaginase production at different fermentation periods. by *Brevundimonas olei* No.15.**

| Carbon sources | Asparaginase activity (IU/ml.) after various fermentation periods (hrs.) | | |
|---|---|---|---|
| | 24 | 48 | 72 |
| **Dextrose** | 391.0 | 322.6 | 155.4 |
| **Sucrose** | 386.2 | 221.9 | 72.3 |
| **Fructose** | 379.3 | 193.5 | 113.4 |
| **Lactose** | 390.1 | 324.6 | 326.5 |
| **Mannitol** | 444.8 | 179.9 | 161.3 |

With regard to the effect of various carbon sources at different fermentation periods on production of L-asparaginase for both *Bacillus subtilis* No.28. and *Bacillus cereus* No. 32., results presented in Table (3) and Table (4) and graphically illustrated by Fig. (5) and (6) showed that, glucose gave the highest enzyme activity being (1283.7 IU/ml.) was obtained after 72 hours for *Bacillus subtilis* No.28. While *Bacillus cereus* No. 32.gave the highest enzyme activity being (757.7 IU/ml.) was obtained after 48 hours fermentation period.

Finally, from the above mentioned results we could be say in generally, glucose was the most prommising one among the tested sugars. Similler results were obtained by **Palaniappan et al,. (2013), Badoei et al., (2015) , Saxena et al,. (2015)** and **Khamna** and **Akira (2016).**

**Table 3. Effect of carbon sources, on asparaginase production at different incubation periods by *Bacillus subtilis* No.28.**

| Carbon Sources | Asparaginase activity (IU/ml) after various incubation periods(hrs.) | | |
|---|---|---|---|
| | 24 | 48 | 72 |
| **Dextrose** | 372.5 | 390.1 | **1283.7** |
| **Sucrose** | 304.0 | 938.6 | 825.2 |
| **Fructose** | 317.7 | 468.3 | 1220.2 |
| **Lactose** | 333.4 | 447.8 | 680.5 |
| **Mannitol** | 392.0 | 514.2 | 457.5 |

**Table 4. Effect of carbon sources, on asparaginase production atdifferent incubation periods. by *Bacillus cereus* No. 32.**

| Carbon sources | Asparaginase activity (IU/ml) after various incubation periods (hrs.) | | |
|---|---|---|---|
| | 24 | 48 | 72 |
| **Dextrose** | 610.1 | 757.7 | 278.6 |
| **Sucrose** | 390.1 | 363.7 | 331.4 |
| **Fructose** | 400.8 | 576.8 | 388.1 |
| **Lactose** | 304.0 | 429.2 | 463.4 |
| **Mannitol** | 432.1 | 523.0 | 283.5 |

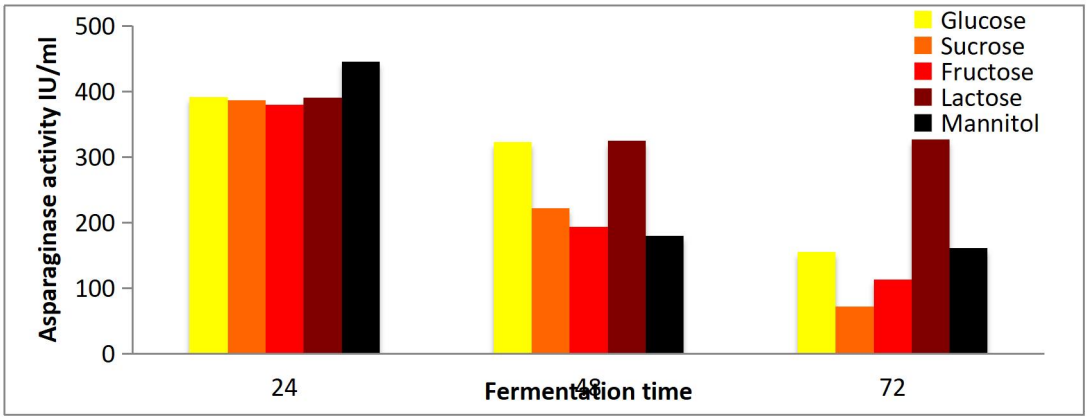


**Fig. 4. Effect of different carbon sources at various fermentation periods on L- asparaginase production by *Brevundimonas olei* .No.15.**

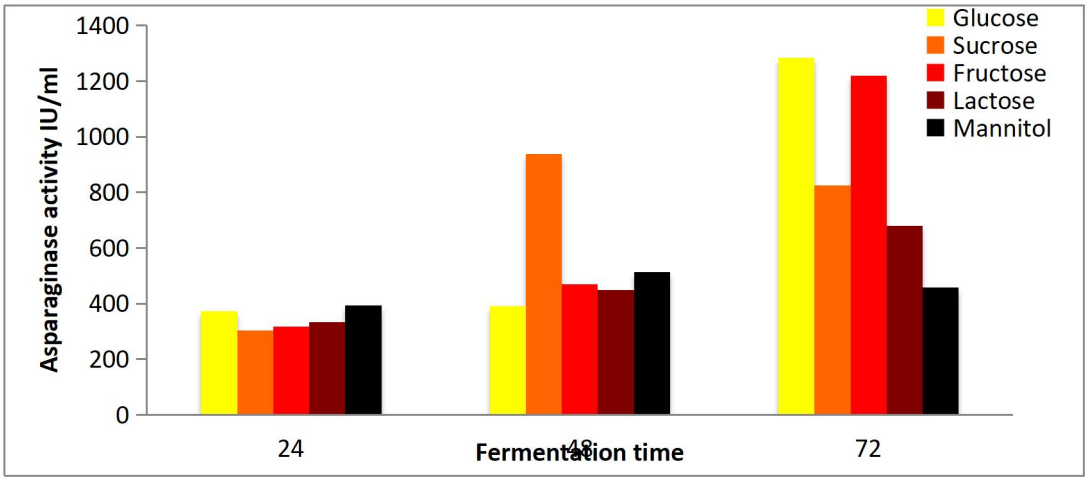


**Fig. 5. Effect of different carbon sources at various fermentation periods on L- asparaginase production by *Bacillus subtilis* No.28.**

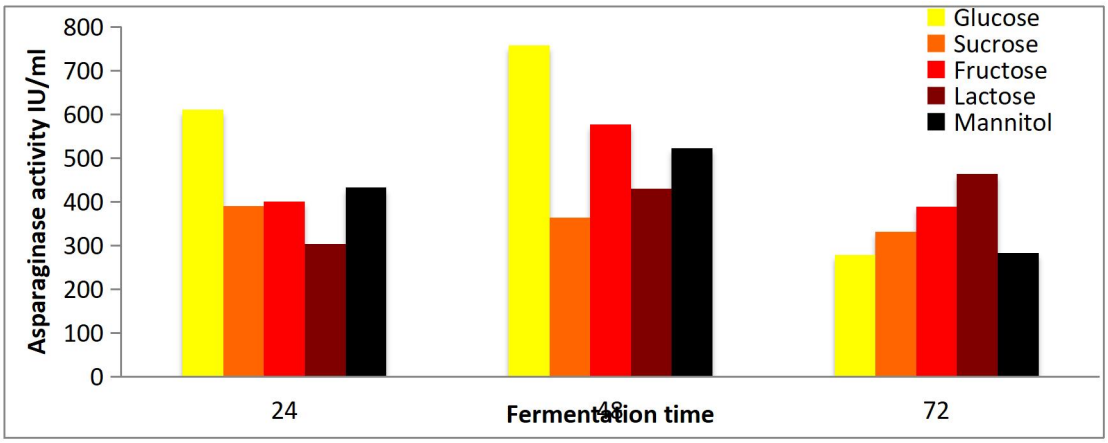


**Fig. 6. Effect of different carbon sources at various fermentation period on L- asparaginase production by *Bacillus cereus* No. 32.**

**Effect of agricultural wastes on production of L-asparaginase at various fermentation periods by tested bacterial strains :**

Due to the fact that may waste-product containing carbohydrate in concentration of 3% or more can be fermented **(Robinson, 1952**). several agricultural for the L-asparaginase synthesis by microorganisms (**Pedreschi et al., (2011), (Kumar et al., 2013)** and **(Dias et al., 2015).** The peels of orange or potato was selected a media substrate for production of L-asparaginase. Therefore an experiment was designed to study the effect of orange and potato peels on production of L-asparaginase at various fermentation periods i.e. (24, 48 and 72 hours) by tested bacterial strains.

Data presented in Table (5) and graphically illustrated by Figs (7), and (8) reveal that, in general, potato peels gave the higher L-asparaginase yield than orange peel for all investigation bacterial strains. The enzyme yield in the media containing potato peel at 72 hours fermentation period gave the highest enzymatic yield being (1070.6 IU/ml.) with *Bacillus subtilis* No.28.

Concerning to orange peels, it was found that the best fermentation period was 72 hours fermentation period which gave the enzymatic activity being (711.7 IU/ml.) and (446.8 IU/ml.) for *Bacillus subtilis* No.28 and *Bacillus cereus* No. 32 respectively. While *Brevundimonas olei* No.15 gave the highest enzyme yield being (361.6 IU/ml.) at 24 hours fermentation period.

According to the aforementioned results, it could be concluded that the tested two wastes differed in their response to bioconversion to L-asparaginase enzyme. Generally it could say *Bacillus subtilis* No.28. and *Bacillus cereus* No.32.were the most efficient strains in the bioconversion of these wastes .

**Table 5. Effect of agricultural wastes on production of L-Asparaginase at various fermentation periods by tested bacterial strains.**

| Fermentation periods (hrs.) | L-Asparaginase activity (IU/ml.) after various fermentation time (hr) | |
|---|---|---|
| | Orange peel | Potato peel |
| ***monas olei* No.15** | | |
| **24** | 364.6 | 246.3 |
| **48** | 346.1 | 487.6 |
| **72** | 244.4 | 215.1 |
| ***Bacillus subtilis*No.28** | | |
| **24** | 404.7 | 283.5 |
| **48** | 434.1 | 355.8 |
| **72** | 711.7 | 1070.6 |
| ***Bacillus cereus*No. 32** | | |
| **24** | 167.1 | 211.1 |
| **48** | 198.4 | 499.6 |
| **72** | 446.8 | 385.2 |

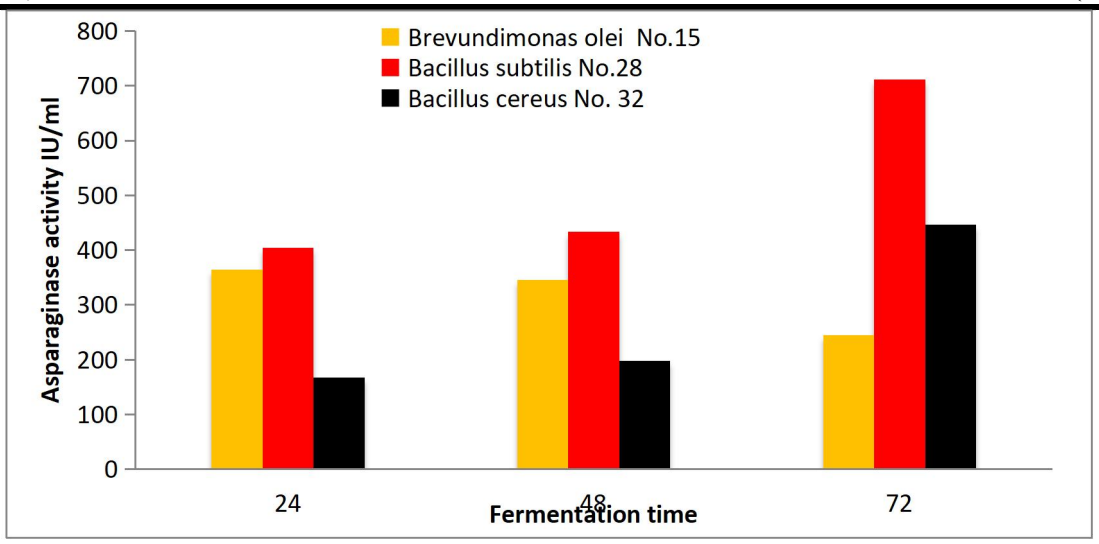


**Fig. 7. Effect of orange peels as a carbon sources and different fermentation periods on L-asparaginase production by tested bacterial strains.**

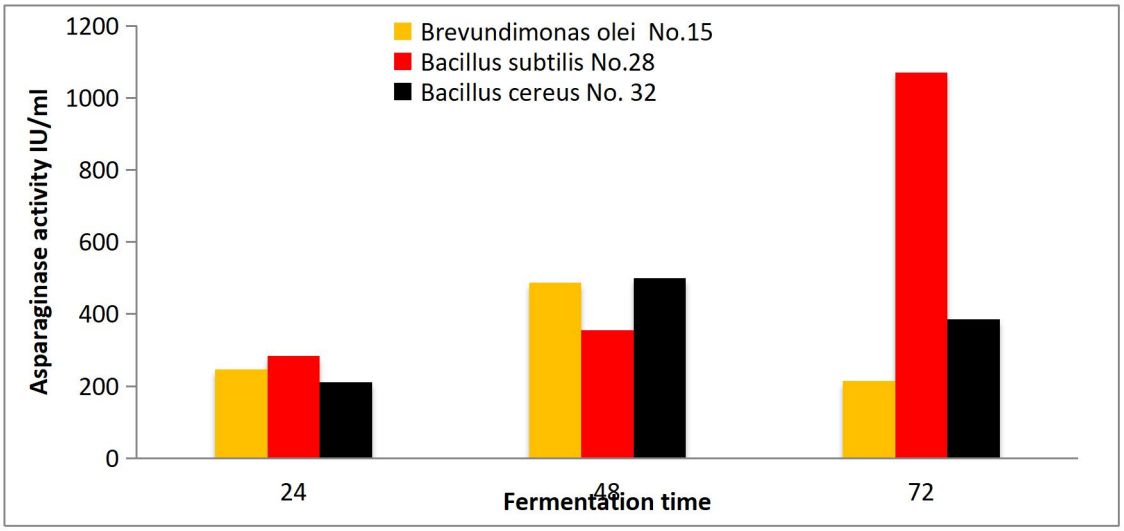


**Fig. 8. Effect of potato peels as a carbon sources and different fermentation periods on asparaginase production by tested bacterial strains.**

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## الظروف المثلى لانتاج انزيم الاسباراجينيز بواسطة السلالات البكتيرية المعزولة من التربة بمحافظة الفيوم

في هذا البحث تم استخدام ثلاث سلالات معزولة والمعرفة مورفولوجيا و وراثيا من التربة بمحافظة الفيوم وهم (*Brevundimonas olei, Bacillus subtilis , Bacillus cereus.*)

وتم دراسة تاثيرالظروف المثلى مثل درجة الحموضة للبيئة – درجة حرارة التحضين و مدة التحضين وكذا مصادر كربون مختلفة وكذا تم الاستفادة من بعض المخلفات الزراعية مثل قشور البطاطس والبرتقال على انتاج الانزيم بواسطة السلالات المختبرة وقد أوضحت النتائج أن بكتريا *Brevundimonas olei,* أعطت اعلى إنتاجية للانزيم على درجة pH :8 و درجة حرارة $30^0$ مئوية. وكان اقل مستوى إنتاجية للانزيم بواسطة السلالة Bacillus subtitles 28 على درجة pH:7 و درجة حرارة 30 . بينما أعطت اعلى إنتاجية pH :9 ودرجة حرارة 40 .

وعند دراسة مصادر الكربون المختلفة (جلوكوز – سكروز-فركتوز – لاكتوز- مانيتول) بتركيز 0.5% على مدد مختلفة 24-48-72) وكانت النتائج المتحصل عليها تشير الى ان *(Brevundimonas olei)* أعطت اعلى إنتاجية للانزيم (444.8 وحدة /مل) بعد 24ساعة. باستخدام المانيتول كمصدر وحيد للكربون يليه الدكستروز 391 وحدة/مل ثم لاكتوز 390 وحدة/مل ثم سكروز 386.2 و الفركتوز 379.3 على التوالي .

وقد تم استخدام قشور البطاطس والبرتقال كمصدر للكربون لانتاج الانزيم وبصفة عامة أوضحت النتائج ان قشور البطاطس أعطت اعلى إنتاجية لكل السلالات البكتيرية المختبرة.

**الكلمات الدالة** : انزيم , اسباراجينيز , الظروف المثلى للإنتاج , المخلفات الزراعية.