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# Over production of cyclosporine A using solid state fermentation by new isolate of *Fusarium oxysporum*

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**Abstract:** Screening for cyclosporine produced by some isolates of *Fusarium* sp was investigated. The study involved testing 7 endophytic isolates of grapevine roots crimson cultivar, two isolates of *Fusarium solani* (Mart.) Sacc, two isolates of *F. oxyspoium* f.sp. *herbemontis* (Tochetto) Gordan and three isolates of *Fusarium oxysporum* Schlechtend from date palm trees. Different agriculture wastes (soybean meal, wheat bran ,wheat straw and Rice bran) were tested under different fermentation conditions such as , fermentation time, glucose concentration as well as solvent used for extraction. The results indicated that the best yield of cyclosporine A ( 855 mg) was obtained by isolate of *Fusarium oxysporum* f.sp. *herbemontis* (no.2) using wheat bran, 3g/l glucose , at 10 days fermentation period and butyl acetate was the best solvent used for extraction.
**Key wards**. Cyclosporine A , solid state fermentation ,*Fusarium* sp.
**Abbreviation** Cyclosporin A (CyA)

## Introduction

Cyclosporins are cyclic 11-membered fungal peptide metabolite which is widely used as a powerful immunosuppressant in transplantation surgery[1]. Different microbial strains that are known to produce cyclosporin A include *Tolypocladiumin flatum, Fusarium solani* and *Neocosmospor auarinfecta*[2,3,4,5].

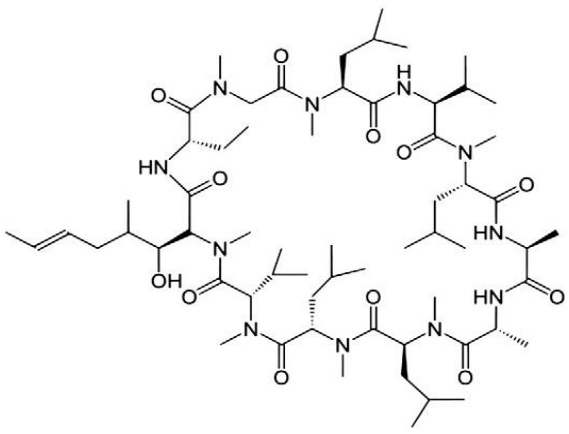


**Fig.(1) structure of CyA**

The production of Cy A is generally carried out by submerged[6,7] fermentation with batch fermentation or internal feeding batch fermentation strategy. Although the internal feeding batch fermentation has a high yield of CyA in contrast to batch fermentation, this pattern almost resulted in the unstable substrate concentration disrupting the biosynthesis of Cy A. However, so far there is no perfect defined continuous fed-batch process for the production process. Endopthytes are fungal pathogens or non-pathogen, which are well known to secrete bioactive compounds as anticancer, ant diabetic, insecticides, antioxadants and immunosuppressive compound such as *Curvularia* sp., *Fusarium subglutinans*, *Fusarium oxysporum* and *Alternaria* sp.[8,9,10,11,12]. Furthermore, endophytic fungi are used as biocontrol agents for controlling plant diseases , stimulation plant growth and establishment its under biotic and a biotic stress[10,13].The present study aimed for production of cyA under solid fermentation technology , selection of the highly potent *Fusarium* isolate.

## Materials and Methods

### 1-Materials

### 1.1- Isolation and identification of *Fusarium* spp. isolates

Samples of root-rot of grapevine Cv crimson, and rotten trunk of date palm trees collected at Kafer El Abida village, Gharbeia Governorate, Egypt were cut into small parts 1cm and surface sterilized by 1% sodium hypochlorite for 3 min then, washed by sterilized distilled water several times then cultured on potato dextrose agar medium (PDA) then incubated at 27C$^{o}$ for 5 days. colony margins were then transferred onto (PDA) as part of the culture purification process by single spore culture technique. Fungal isolates were identified according to [14,15].Meanwhile two isolates of *Fusarium oxysporum* f.sp. *herbemontis* has been isolated and identified previous studies[14].The fungal strain was maintained on (malt extract 2.5% yeast extract 0.4%, agar 2%) (MY medium), stored at 4°C and were monthly regenerated.

### 1.2-Chemicals

The authentic CyA was provided by Sigma company. All the other chemicals were laboratory reagents obtained from Merck and the solvents used are HPLC grade.

### 2-Experimental methods

### 2.1-Inoculum's preparation

The used inocula was obtained from14 days old slant, 5 ml of sterile saline containing 0.1% Tween 20 was added and the spores conc. (2.5 x $10^{7}$spores/ml) were scrapped from the slant and mixed thoroughly. 5 ml from the previously prepared suspension was used to inoculation each flask[16].

### 2.1-Preparation of solid substrate

According to the modified method of [16] where,50 g. of the agriculture waste used as the solid substrate for the production was taken in a 250 ml flask and 70 ml of 0.2 N HCl was added and mixed well and was autoclaved for 60 min at 121$^{o}$C/15 . The flasks were then cooled to room temperature.

### 2.2-Fermentation process

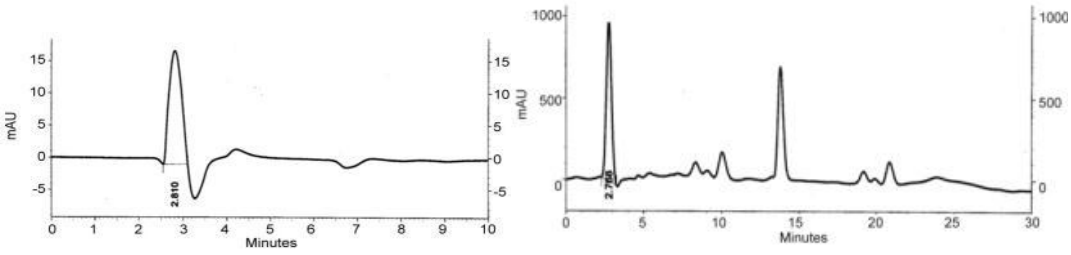
Erlenmeyer flasks(250 ml) containing sterilized waste (50g) were inculcated by 5ml spore suspension 2.5 x $10^{7}$ spore /ml of each tested *Fusarium* strains, flakes were incubated at 30$^{o}$C for 14 days[17].

### 2.3-Extraction of Cy A

At the end of the fermentation 100ml the butyl acetate to each flask, in the ratio (1:2 w/v ration) and kept in a shaker at room temperature for overnight extraction. This was then altered through a muslin cloth and extracted once more with butyl acetate. The combined extracts were altered through a Whatman no 1 paper to give a clear, dark brown colored extract.

### 2.4.Analysis of CyA

The qualitative analysis of CyA was achieved by using TLC plates according to the method described b y [18,19,20] .The HPLC conditions were 70/30 (V/V) of acetonitrile and water as the mobile phase, 205 nm of ultraviolet detective wavelength, 1.5 mL/ min of flow rate, 70°C of column temperature and 20 µL of sample size. The CyA was identified and quantified based on the similarity of retention time with that of standard Cy A which was used for the calibration of the system.




**(a)** **(b)**

**Fig. (2) HPLC analysis (a ) the authentic Cy A (b) sample**

### Statistical analysis

Data obtained was statistical analysis using Duncan's multiple range test according to [21].

## Results and Discussion

### 1-Investigation of different *Fusarium* isolates on CyA production

Seven isolates of *Fusarium* were tested for production CyA. Four isolates were isolated from roots of diseased grapevine plants by wilt and root rot diseases of Cv. cirmson, they are 2 isolates of *Fusarium solani* and2*Fusarium oxysporum* f. sp.*herbamontis* and3 isolates of *Fusarium oxysporum* of date palm trunk. All the isolated strains were showed to be CyA producer but varied in their productivity. The results in Table (1) showed that *F. oxysporum* f.sp.*herbamontis* was the best cyA producer since, it gave (546.0 mg).The lowest yield (231.0 mg) was obtained by *F. oxysporum* (no.1).The cyclosporin A productivity was recorded by *Fusarium* sp. was stated by [18].

**Table (1) Screening for the most potent *Fusarium* spp. isolates on cyclosporine A production**

| Microbial strains | Consumed waste weight (g) | CyA production (u mg) | Specific production mg/gwt |
|---|---|---|---|
| *Fusarium solani* (no.1) | 1.44 | 324 | 225.00g |
| *Fusarium solani* (no.2) | 1.34 | 533 | 397.76b |
| *F.oxysporum* ( no.1) | 1.54 | 231 | 23251f |
| *F.oxysporum* (no. 2) | 1.43 | 367 | 256.64f |
| *F.oxysporum* (no.3) | 1.56 | 421 | 269.87e |
| *F. oxy*.f.sp.*herbamontis* (no.1) | 1.43 | 446 | 381.81c |
| *F. oxy*.f.sp.*herbamontis* ( no .2) | 1.44 | 478 | 479.44a |

Mean within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P$>0.05)

### 2-Application of some agriculture wastes on CyA production

The effect of different agriculture wastes (soybean meal, wheat bran, wheat straw, and rice bran) was investigated .The results presented in Fig.(3) showed that the best cy A production(564 .0 mg) was obtained using of wheat bran. Therefore, it was well selected as the substrate to continue the following experiments. On the other hand, the lowest CyA (234 mg) was obtained by using wheat straw. These results correlated with that

obtained by[16] who stated that the acid hydrolysis of wheat bran led to amino acid take part in cyclosporine biosynthesis.

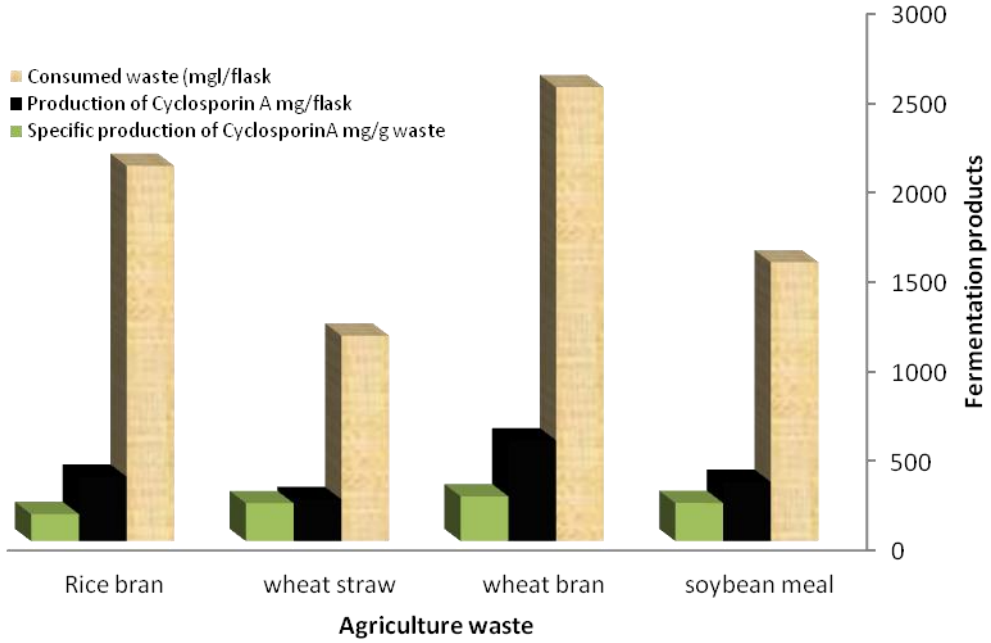



**Fig.(3) Effect of different agriculture wastes**

### 3- Solvents used for CyA extraction

Different solvents(acetone , methanol, ethyl acetate, dichlochoroethane, chloroform and butyl acetate ) were investigated for cy A extraction. The data presented in Fig.(4 ) revealed that the best CyA output (564.0 mg ) were obtained by using butyl acetate. On the other hand, the lowest CyA yields (100, and 123.0 mg) were obtained by using distilled water and acetone, respectively. The statistical data showed that the solvent for extraction is significant in the production process and also showed that no correlation was observed between the consumed dry weight consumed and the solvent type while, great significant was notice on the productivity of cyA and the type of the solvent used. These results correlated with that stated by [19-20].

### 4-Inoculum concentrations

Different inoculums concentrations of the used strain were tested. The results presented in Table (2) showed that the maximum output (677 mg ) was obtained by using 2.5 x$10^7$inoculum.Meanwhile, the other values above and below this concentration showed remarkable decrease in the cy A yields. The statistical data revealed that the effect of inoculums concentration is highly significant on the productivity and on the waste consumption rate . On the other hand, no significance was recorded at the concentration 1X$10^7$ spore /ml. In accordance to these results [16,21,22].

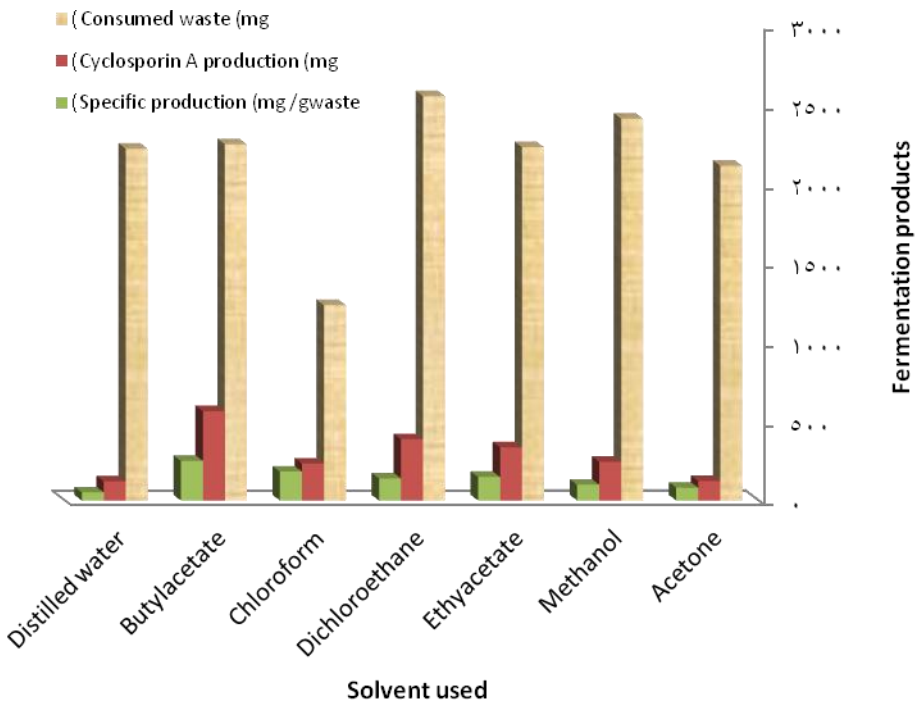



**Fig.(4) Effect of different solvents used in cyA extraction**

**Table (2)Effect of different inoculum concentration oncyA production**

| **Inoculum concentration** | **Consumed waste weight (g)** | **Cyclosporine A (mg)** | **Specific production (mg/g)** |
|---|---|---|---|
| 1x10 $^{7}$ | 1.34 | 234 | 174.62 |
| | 1.32 | 243 | 184.09 |
| | 1.32 | 255 | 193.18 |
| | | | 184.0 c |
| 2x10$^{7}$ | 1.55 | 340 | 219.35 |
| | 1.57 | 345 | 219.74 |
| | 1.56 | 355 | 227.56 |
| | | | 222.3b |
| 2.5x10$^{7}$ | 2.21 | 546 | 247.05 |
| | 2.32 | 564 | 243.10 |
| | 2.20 | 677 | 262.27 |
| | | | 250.6 a |
| 3x10$^{7}$ | 1.78 | 378 | 212.35 |
| | 1.56 | 367 | 235.25 |
| | 1.77 | 365 | 201.12 |
| | | | 216.0b |
| 4x10$^{7}$ | 1.21 | 221 | 182.64 |
| | 1.22 | 213 | 174.59 |
| | 1.23 | 233 | 189.34 |
| | | | 182.0 c |
| 5x10$^{7}$ | 1.33 | 123 | 92.48 |
| | 1.34 | 133 | 99.25 |
| | 1.43 | 130 | 90.90 |
| | | | 940.0 d |

Mean within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P$>0.05)

**5-Fermentation time course on cyA production**

In the present experiment the effect of different fermentation time (3,5,8,10,12,14 day) on the production process were investigated. Data showed in Fig.(5) indicated that the maximum CyA output (677 mg) was obtained at 10 days fermentation time, these results disagree with that obtained by [22,23,24] who showed that the best cy A (546.0 mg)was obtained at 14 days. The productivity was noticeably decreased at the longer fermentation time .The statistical data revealed that significant difference>0.05 at time 8 and 10 days .

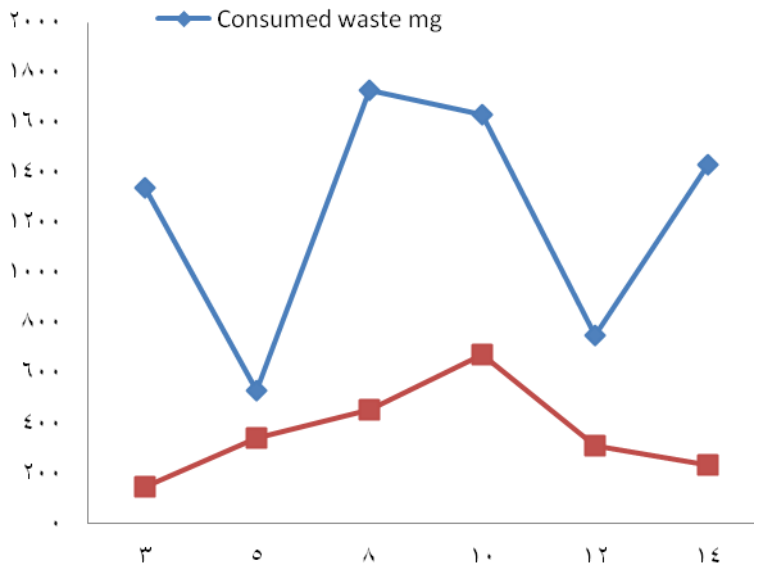



**Fig.(5) Effect of different fermentation time on the production of cyclosporin A**

**6- glucose concentration**

The effect of different doses of glucose (1,2,3,4,5,and10g/l) addition to the fermentation medium was tested. The results presented in Table (3) revealed that the best Cy A yield ( 853.0 mg) was obtained by the addition of 3 g glucose to the fermentation medium. On the hand, the concentrations above and below 3g/L produce a remarkable decrease on the production of cyclosporine A by the selected strain.

**Table (3) Effect glucose addition on the production process**

| Glucose conc. (g/L) | Consumed waste weight( g) | Cyclosporine A (mg/l) | Specific production (mg/g) |
|---|---|---|---|
| 1 | 1.67 | 349 | 208.98 |
| | 1.56 | 345 | 221.15 |
| | 1.45 | 356 | 245.51 |
| | | | 225.3 c |
| 2 | 2.21 | 546 | 247.05 |
| | 2.32 | 564 | 243.10 |
| | 2.20 | 577 | 262.27 |
| | | | 250.6 b |
| 3 | 2.21 | 853 | 385.97 |
| | 2.22 | 845 | 380.63 |
| | 2.13 | 855 | 401.40 |
| | | | 389.3 a |
| 4 | 2.33 | 445 | 190.98 |
| | 2.31 | 456 | 197.40 |
| | 2.21 | 453 | 204.97 |
| | | | 197.6 d |
| 5 | 1.34 | 312 | 232.83 |
| | 1.44 | 312 | 222.91 |
| | 1.32 | 316 | 239.93 |
| | | | 232.0c |
| 10 | 1.55 | 234 | 150.96 |
| | 1.67 | 244 | 146.10 |
| | 161 | 231 | 143.47 |
| | | | 146.6 e |

Mean within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P$>0.05)

## Conclusion

There are many publications for production of CyA using submerged fermentation; very few reports are available on solid state fermentation (SSF) of CyA. This study gave highlight about the productivity and it appeared that (SSF) is considered to be a valuable method due to the low costing .The results showed that the priority of using wheat bran as agriculture waste for CyA production as well as butyl acetate is recommended for the extraction. Meanwhile, the productivity was enhanced by the addition of glucose 3g to the agriculture waste and the maximum CyA productivity was achieved at 10 days fermentation period..

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