



## Characterization and Application of *Aspergillus tubingensis* USMI03 RPF10 Fermented Solution as a Nutrient Source for Microbial Growth

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### Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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### ABSTRACT

The fermented solution of this strain contains the highest concentration of citric acid, indole acetic acid (IAA) and elements as well as phosphatase activity. Using the *Aspergillus tubingensis* USMI03 RPF10 fermented solution as a medium led to decrease in the growth rate of tested bacterial isolates (*Bacillus subtilis*, *Pseudomonas fluorescens* and *Escherichia coli*) and fungal isolates (*Aspergillus* sp. and *Penicillium* sp.) except *Trichoderma viride* which gave the same growth on fermented solution treatments compared to control. Whereas the tested yeast isolates (*Candida olivera* and *Saccharomyces cerevisiae*) recorded the same growth rate on fermented solution treatments as whole media and higher growth rate on fermented solution + glucose treatment, compared to control. Also, the latter treatment resulting in higher growth of some tested bacteria (*B. subtilis* and *P. fluorescens*) and the same growth rate of other bacteria (*E. coli*), compared to synthetic media. The growth of *Rhizopus* sp. decreased on fermented solution treatments than control. It is suggested that, using *A. tubingensis* USMI03 RPF10 fermented solution as a whole medium or as a mineral could be used as a source for microbial growth which varied from one microorganism to another.

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## 1. INTRODUCTION

Phosphorus (P) is the most important element in the nutrition of plants, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plants, including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration [1]. During rock phosphate biosolubilization, certain elements are released as well as organic acids and growth promoting produced [2]. A phytase (myo-inositol hexakisphosphate phosphohydrolase) is one the type of phosphatase that catalyzes the hydrolysis of phytic acid and released P from inorganic phosphate [3,4,5]. Phosphatase produced by microorganisms play a vital role in phosphate solubilization [6,7]. The organic acids produced by many P-solubilizers, for example, bacterial cultures or fungi, in a natural environment or under *in vitro* conditions chelate mineral ions or decreased pH to bring P into solution [8]. The type of acid produced is dependent on the microorganism. Gluconic acid and 2-ketogluconic acid are produced as the principal organic acid by phosphate solubilizing bacteria and fungi such as *Pseudomonas fluorescens*, *Aspergillus niger* and *Penicillium canescens* [9,10]. Oxalic and citric acids are produced by *Arthrobacter* sp., *Azotobacter* sp., *Enterobacter* sp., *Aspergillus niger*, *A. awamori*, *Penicillium bilaiae* and *P. purpurogenum* [11,12,10]. Among all phosphate solubilizing microorganisms, fungi are more efficient to solubilize phosphate than bacteria in solid and liquid media [13]. The most efficient fungal species were *Aspergillus niger*, *A. tubingensis*, *A. terreus* and *Penicillium citrinum* [14,15]. Hence, the present investigation aimed to identify the rock phosphate solubilizing fungi. Therefore, characterization of the fermented solution produced from tested fungi and application the fermented solution produced as a mineral source for microbial growth were investigated.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms

The tested organisms used in this investigation were; *Bacillus subtilis*, *Pseudomonas fluorescens*, *Escherichia coli*, *Candida olivera*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Penicillium* sp., *Trichoderma viride* and *Rhizopus*

sp. were obtained from Department of Microbiology, Fac. of Agric., Ain shams Univ. Cairo, Egypt. The strain *Aspergillus tubingensis* USMI03 RPf10 was used as solubilizer of phosphate in this investigation. This strain was previously isolated from rock phosphate and identified using pheno and genotypes characteristics [16,17]. It was maintained by transferring at regular intervals on malt agar slants [18] and incubated at 30°C for 24 h. Slants were kept at 4°C until used.

### 2.2 Media Used

**Malt agar medium:** EMCC [18], was used for maintenance and preservation of tested fungal cultures. It has the following composition (gL<sup>-1</sup>): malt extract, 30; agar, 20 and adjusted to pH 5.0.

**Czapek Dox broth medium [19]:** This medium was used for maintenance of fungal strains. Its composition was as follows (gL<sup>-1</sup>): sucrose, 30; NaNO<sub>3</sub>, 3; K<sub>2</sub>HPO<sub>4</sub>, 1; KCl, 0.5; FeSO<sub>4</sub>, 0.1; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 and adjusted to pH 5.0.

**Nutrient agar medium:** Difco [20], was used for maintenance and preservation of tested bacterial cultures. It had the following composition (gL<sup>-1</sup>): beef extract, 3.0; peptone, 5.0 and adjusted to pH 7.0.

**Yeast malt extract broth medium (YMG) [21]:** It was used for maintenance of yeast cultures. It had the following composition (gL<sup>-1</sup>): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10 and adjusted to pH 6.0.

### 2.3 Preparation of *Aspergillus tubingensis* Rf10 Fermented Solution

It was carried out in 250 ml plugged Erlenmeyer flask, containing 100 ml sterile modified fermentation medium by Abou-Taleb et al. [17] [(gL<sup>-1</sup>): sugar beet waste, 30; rock phosphate, 10 and adjusted to pH 7.0] and inoculated with 3% spore suspensions (3.18×10<sup>8</sup> spores ml<sup>-1</sup>) for *A. tubingensis* USMI03 RPf10 strain which was incubated at 30°C on rotary shaker at 150 rpm for 12 days. The fermented medium was filtered through filter paper No.1, the supernatant was used as the fermented solution.

## 2.4 A. *tubingensis* USMI03 Rf10 Fermented Solution as a Nutrient Source for Microbial Growth

This experiment was constructed to study the growth behavior of tested bacterial, yeast and fungal isolates grown on *A. tubingensis* USMI03 Rf10 fermented solution as whole medium and as a mineral source, after supplemented with carbon source. The control treatments were used to cultivate the tested bacterial, yeast and fungal isolates on nutrient broth, YMG and malt or Czapek Dox broth media, respectively. The propagation was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium. These flasks were inoculated with 1 ml standard inoculum ( $1.63 - 3.8 \times 10^8$  CFU ml<sup>-1</sup>). The inoculated flasks were incubated for 36h, 72h and 6 days, respectively at 30°C using rotary shaker at 150 rpm. Samples (10 ml) were taken from the growing cultures periodically every 6-12 hours for bacterial & yeast and 2 days for fungal growth to determine, respectively. The relation between incubation time and microbial growth was plotted using an Excel program. All parameters of microbial growth (specific growth rate, doubling time & multiplication rate) and correlation coefficient between microbial growth and incubation period were calculated.

## 2.5 Analytical Methods

Growth of tested bacteria was determined the optical density of growth spectro-photometrically at 620 nm (Unico S2100 series UV/Vis). Fungal cells dry weight was determined by filtration using Whatman filter paper No.1 washed twice, dried at 70°C until constant weight. Total sugar was determined by phenol-sulfuric acid method [22]. The indole acetic acid (IAA) was quantified in fermented solution, using a colorimetric technique by Salkowski reagent as described (Glickmann and Dessaux) [23]. Samples were mixed 1:1 (v<sup>-1</sup>) with Salkowski reagent (12 gL<sup>-1</sup> FeCl<sub>3</sub>, 7.9 M H<sub>2</sub>SO<sub>4</sub>) and incubated for 30 min in darkness. The resulting absorbance was determined at 530 nm. IAA concentration was calculated using the standard curve which constructed by using IAA. Citric acid content was determined in supernatant by a spectrophotometric method using pyridine and acetic anhydride reagents [24]. Concentration of citric acid were calculated according to the standard curve of citric acid. The activity of acid phosphatase was determined by the acid

phosphatase (ACP) Cod. AP013 Kit obtained from Al-gomhoria Co., Cario, Egypt [25].

## 2.6 Growth Kinetics Calculation

The specific growth rate ( $\mu$ ) and doubling time ( $t_d$ ) were calculated from the exponential phase according to Painter and Marr [26] using the following equation: Specific growth rate ( $h^{-1}$ )  $\mu = (\ln x - \ln x_0) (t - t_0)^{-1}$  and doubling time ( $t_d$ ) ( $h$ ) =  $\ln(2) (\mu)^{-1}$ .

where,  $\mu$  = Specific growth rate ( $h^{-1}$ ),  $x_0$  = Growth at  $t_0$  time ( $g L^{-1}$ ),  $X$  = Growth after  $t$  time ( $g L^{-1}$ ),  $t_0$  = Time in hours at the beginning of log phase and  $t$  = Time in hours at the end of log phase.

Multiplication rate (MR) [27] was calculated according to the following equation:  $MR = 1/(t_d)^{-1}$ .

## 2.7 Statistical Analysis

The correlation coefficient was analyzed with Microsoft Office Excel 2013.

## 3. RESULTS AND DISCUSSION

### 3.1 Some Properties of Fermented Solutions Produced by *A. tubingensis* USMI03 Rf10

In the previous study by Abou-Taleb et al. [17], the fermented solution produced by *A. tubingensis* USMI03 Rf10, contained high concentrations of organic acids (citric acid 543.39  $\mu g ml^{-1}$ ), growth promoting (IAA 11.96  $mg L^{-1}$ ) and high phosphatase activity (971.0  $UL^{-1}$ ). Regarding elements analysis of fermented solution produced by *A. tubingensis* USMI03 Rf10, the results listed in Table 1 revealed that the highest element concentration of fermented solution was attained by K followed by N and P being 369, 215 and 196.2  $mg L^{-1}$ , respectively. The concentration of other elements included Fe, Mn, Zn and Cu ranged from 0.13 to 0.7  $mg L^{-1}$ .

Similar results were reported by Vassileva et al. [28] sugar beet waste was the best waste for phosphate solubilization by *Aspergillus niger* which increased fungal growth, organic acids formation, phosphate-soluble and rate of mineralization which ranged from 56% to 69%. As recorded with many investigators, *A. niger* was better phosphate solubilization (328  $\mu g mL^{-1}$ ), IAA production (85  $\mu g mL^{-1}$ ) in medium than other fungi.

**Table 1. Chemical analysis of fermented solution produced by *A. tubingensis* USMI03 RPF10**

Elements	Fermented solution (mgL <sup>-1</sup> )
N	215
P	196.2
K	369
Fe	0.26
Mn	0.70
Zn	0.27
Cu	0.13

Oxalic and citric acids were released by *A. niger* and *A. calvatus* during solubilization of phosphate. Whereas, the main acids produced by *A. niger* for stimulating the solubilization of insoluble phosphate were oxalic and lactic acids [29,30,31]. The phosphate enzyme activities of the most efficient phosphate solubilizing *Aspergillus* sp. isolates ranged from 0.029 to 0.102 U mL<sup>-1</sup> min<sup>-1</sup> for acid phosphatase at pH 6.5 and from 0.022 to 0.102 U mL<sup>-1</sup> min<sup>-1</sup> for alkaline phosphatase at pH 11. However, the highest activity of phytase was recorded at pH 6.0 by *Aspergillus* sp. 5990 and *A. niger* St-6. Also *A. awamori* produced phosphatase at pH ranged from 4 to 10 and maximal activity of enzyme was achieved at pH 6. Furthermore, there was relationship between the formation of organic acids (titrable acid) and activity of phosphatase for phosphate solubilization [32,33,34,35].

It can be concluded that, the fermented solution produced by *A. tubingensis* USMI03 RPF10 contained high concentrations of organic acids, growth promoting (IAA) and micro & macro elements as well as high phosphatase activity. So, this solution will be used as a nutrient source in agriculture or laboratory applications.

### 3.2 Application of *A. tubingensis* USMI03 RPF10 Fermented Solution

#### 3.2.1 As a nutrient solution for microbial growth

In order to evaluate the efficiency of *A. tubingensis* USMI03 RPF10 fermented solution as a nutrient medium for microbial growth, it was used as a whole medium and as a mineral source after supplemented with carbon source. Bacterial, yeast and fungal cells were cultivated in nutrient broth, YMG and malt extract or Czapek's Dox media, respectively as control for the first treatment and after supplemented with carbon source for the second treatment of fermented solution.

Data presented in Fig. (1 a, b) show that the optical density of growth and growth parameters of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Escherichia coli* during 36 h incubation period in tested nutrient broth medium and fermented solution with or without glucose as carbon source. Results clearly showed that all tested bacterial strains grew exponentially during the first 24 h in all tested media except *P. fluorescens*, which increased the log phase to 30 h at nutrient glucose medium and fermented solution as whole medium. The shortest log period being 12 h was recorded by *B. subtilis* on fermented solution supplemented with glucose. The highest bacterial growth was attained at the end of log phase of all treatments. Both fermented solution treatments gave higher growth (as O.D) than control treatments which recorded the highest values by *B. subtilis* followed by *P. fluorescens* and *E. coli* on fermented solution supplemented with glucose being 4.09, 2.21 and 1.13 after 12, 24 and 24 h incubation period, respectively (Fig. 1a). Which was the favorable of fermented. solution by tested bacterial isolates due to the presence of mineral nutrients and growth factors play an important role in enhancement the microbial growth.

These results are accordance with Leveau and Lindow [36] who reported that *P. putida* 1290R grew well on minimal medium containing IAA, which used IAA as a sole carbon, nitrogen and energy sources.

With respect to growth parameters which were calculated from a growth curve of different bacterial cultures, data in Fig. (1b) clearly show that the specific growth rate values of tested bacteria grown on fermented solution as whole medium were lower than that grown on nutrient broth medium. In contrast, fermented solution supplemented with glucose recorded higher specific growth rate ( $\mu$ ) of *B. subtilis* and *P. fluorescens* than control (nutrient glucose medium). The same value of specific growth rate

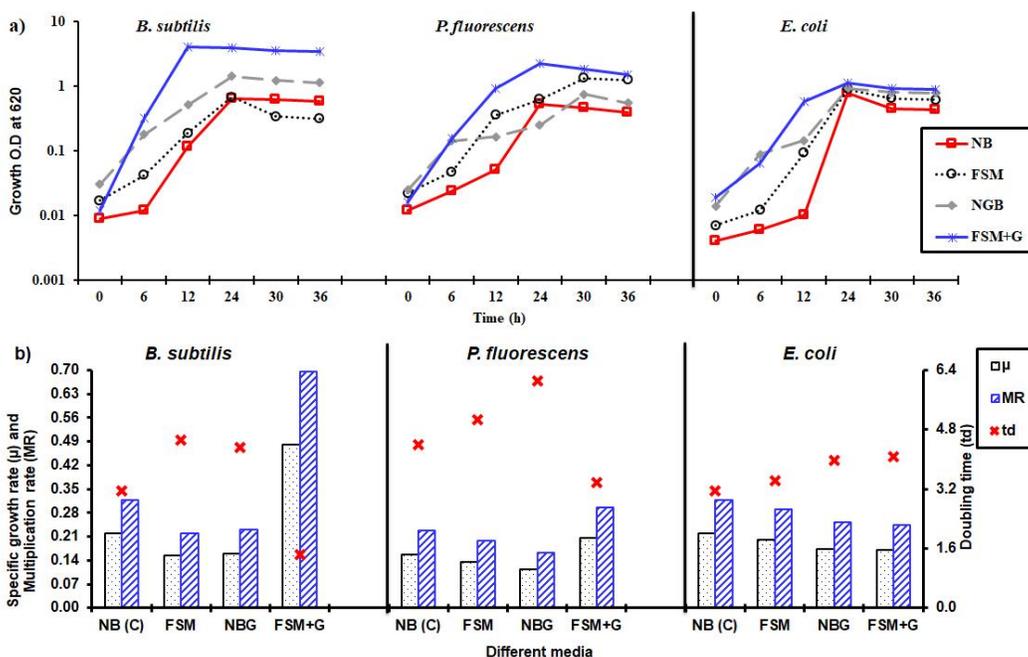
( $0.17 \text{ h}^{-1}$ ) was obtained by *E. coli* on both fermented solution + glucose and control treatments.

A high positive correlation coefficient ( $r$ ) was recorded between the growth of bacterial isolates and incubation period for all treatments. The values of ( $r$ ) of fermented solution treatments were higher than recorded by control treatments except *B. subtilis*  $r$  values for all tested bacteria on all treatments which ranged between 0.69 and 0.96. At fermented solution treatments, the  $r$  value of fermented solution as mineral source ( $r=0.74$  &  $0.93$ ) was higher than fermented as a whole medium ( $r=0.69$  &  $0.89$ ) for *B. subtilis* and *E. coli*. In contrast,  $r$  value of *P. fluorescens* growth on fermented as a whole medium ( $r=0.96$ ) was higher than fermented solution as mineral source ( $r=0.85$ ).

Both tested yeast isolates (*Candida olivera* and *Saccharomyces cerevisiae*) were grown on yeast extract malt extract broth medium and fermented solution with or without glucose as carbon source for 72 h. Data presented in Fig. (2 a, b) show that

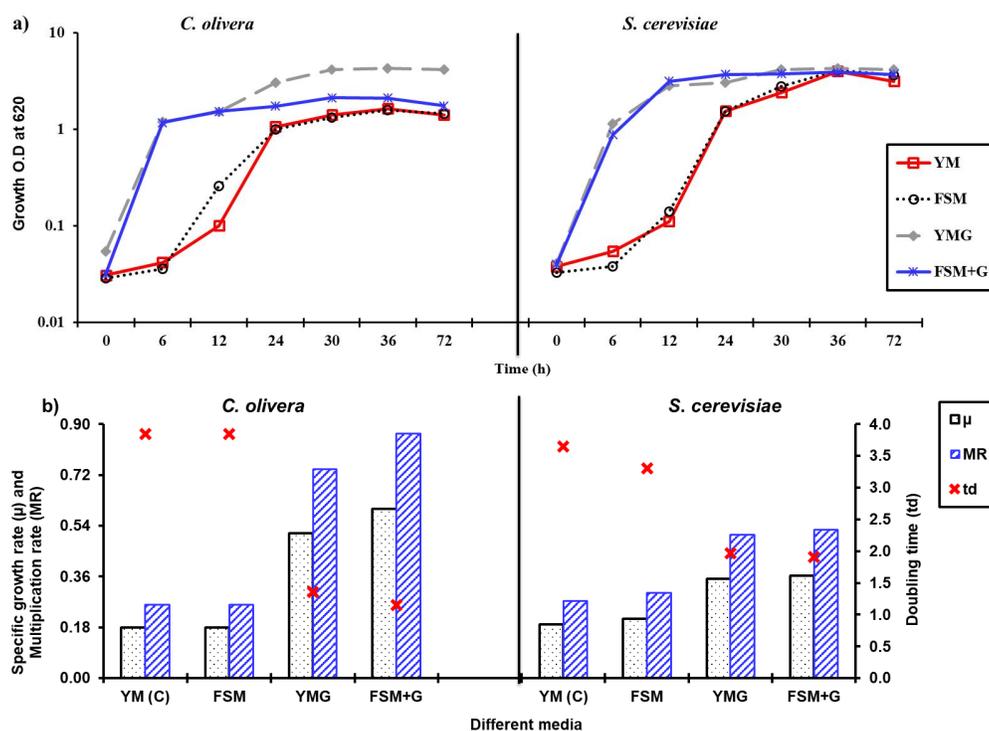
both isolates grew exponentially during the first 6 h of incubation on both media of yeast extract malt extract+ glucose (YMG) and fermented solution supplemented with glucose. Whereas, it increased up to 24h on yeast extract malt extract medium (YM) and fermented solution as a whole medium (Fig. 2a). These yeast isolates recoded the lag period during the first 6 h on nutrient and fermented solution as a whole medium. At fermented solution glucose treatment, *C. olivera* and *S. cerevisiae*, recorded the highest specific growth rate ( $0.60 \text{ h}^{-1}$  &  $0.36 \text{ h}^{-1}$ ) and lowest doubling time (1.16 & 1.91 h). The corresponding figure of multiplication rate was 0.87 for the first isolate and 0.52 for the second isolates.

The lowest specific growth rate and highest  $t_d$  values being  $0.18 \text{ h}^{-1}$  and 3.85 h for *C. olivera* and  $0.19$  &  $0.21 \text{ h}^{-1}$  and 3.65 & 3.30 h for *S. cerevisiae* at two treatments, respectively (Fig. 2b). A high positive correlation coefficient ( $r$ ) was recorded between the growth of yeast isolates and incubation period for all treatments. The values of ( $r$ ) of fermented solution as a whole



**Fig. 1. Growth curves and parameters of tested bacterial cultures grown on nutrient broth and nutrient glucose broth as well as *A. tubingenis* USMI03 *RPf10* fermented solution as a whole medium or supplemented with glucose during 36 h at 30°C using shake flasks as a batch culture**

a) Growth curves b) Growth parameters (Specific growth rate ( $\text{h}^{-1}$ ), multiplication rate (MR) and doubling time ( $t_d$ )); NB= Nutrient broth, FSM= Fermented solution as whole medium, NGB= Nutrient glucose, FSM+G= Fermented solution as whole medium +Glucose, C= control



**Fig. 2. Growth curves and parameters of tested yeast cultures on yeast malt broth and yeast extract malt extract broth as well as *A. tubingensis* USMI03 RPF10 fermented solution as a whole medium or supplemented with glucose during 72 h at 30°C using shake flasks as a batch culture**

a) Growth curves b) Growth parameters (Specific growth rate ( $h^{-1}$ ), multiplication rate (MR) and doubling time ( $t_d$ ))  
 YM= Yeast extract malt extract broth, FSM= Fermented solution as whole medium, YMG= Yeast malt extract broth+ glucose, FSM+G= Fermented solution as whole medium +Glucose, C= control

medium was higher than recorded by control treatments. At fermented solution treatments, the range of ( $r$ ) value of fermented solution as a whole medium was higher than fermented solution as a mineral source and ranged between 0.83 and 0.85 for *C. olivera* and *S. cerevisiae*.

With regarded to the growth behavior of *A. niger*, *Penicillium* sp., *T. viride* and *Rhizopus* sp. on all tested treatments, data illustrated at Fig. (3 a, b) indicated that all tested fungal isolates grew exponentially during the first 2-4 days of incubation at all treatments and recorded the highest growth dry weight at the end of this period, where after a decreased occurred with increasing the incubation period to 6 days. At the end of log phase, all tested fungal isolates recorded the growth dry weight on fermented solution as whole medium lower than malt extract medium, whereas using the fermented solution as mineral source (supplemented with sucrose), increased the growth of *A. niger*, *Penicillium* sp. and *T. viride* about 1.94, 1.53 & 1.33-fold

compared to that obtained by Czapek's Dox medium after 4 days of incubation and increased the growth as *Rhizopus* sp. about 1.13-fold after 6 days of incubation. The growth parameters of tested fungal isolates gave the same trend, where the specific growth rate values of both *Penicillium* sp. and *Rhizopus* sp. on fermented solution treatments were lower than control treatments. *T. viride* recorded the same value of specific growth rate (0.01) on all treatments of fermented solution and control. Also, *A. niger* gave the same specific growth rate (0.03  $h^{-1}$ ) on Czapek's Dox and malt extract media but it decreased on fermented solution as a whole medium and supplemented medium with sucrose than control (malt extract and Czapek's Dox media).

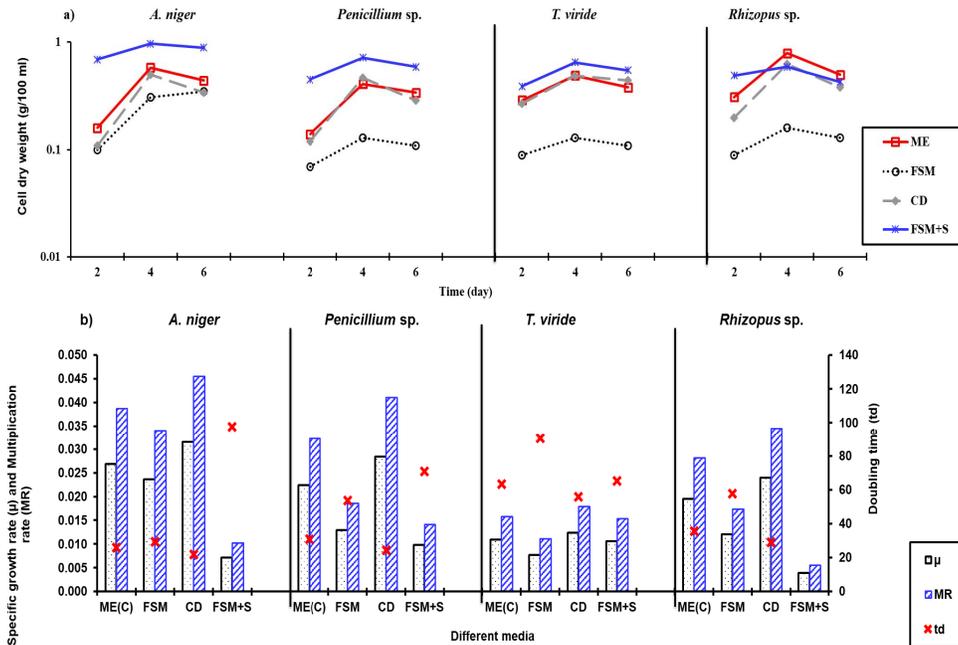
A higher positive correlation coefficient ( $r$ ) was recorded between fungal cell dry weight and incubation period at fermented solutions treatments ( $r$  ranged from 0.7- 0.9) than control ( $r$  ranged from 0.6-0.7) for *A. niger*. No remarkable

change in (r) value was observed by the growth of *Penicillium* sp. on malt extract medium as control and fermented solution as whole medium (r=0.7) and fermented solution + sucrose and Czapek's Dox medium (r= 0.5). While (r) value of *T. viride* grown on fermented solution supplemented with sucrose had a higher positive impact (r=0.7) than other treatments (moderate positive correlation coefficient ranged from 0.4-0.6). In case of *Rhizopus* sp. (r) values were moderate positive in all media except on fermented solution supplemented with sucrose medium, which was moderate negative correlation coefficient (r=-0.4). Also, it was noticed that the (r) values at fermented solution as whole medium were higher than fermented solution supplemented with sucrose.

The plant growth hormone (cytokinin) can increase the growth rate of *A. oryzae*, *A. terreus*, *A. niger* and *Alternaria alternata* [37]. Addition of a carbon source in fermented solutions was increased the microbial growth due being its main nutrients for growth and energy. Fermented

solutions containing macro- and micro-nutrients play an important role in the synthesis of nucleic acid and cellular energy transfers. In addition, the growth rate of *A. flavus* was stimulated in culture medium supplemented with sugar as the sole carbon source such as glucose, fructose, sucrose, or sorbitol [37,1,38].

Generally, it can be concluded from the previous results that using the *A. tubingensis* USMI03 RPf10 fermented solution as a whole medium, led to a decrease in the growth rate of tested yeast and fungal isolates, except *S. cerevisiae*, which gave the same growth on fermented solution treatment, compared to the control. In contrast, the growth of all tested bacteria increased on fermented solution as a whole medium, compared to the control. Also, the latter treatment (fermented solution supplemented with glucose or sucrose) resulted in high growth of some tested bacteria (*B. subtilis* and *P. fluorescens*), yeast (*S. cerevisiae*) and fungi (*A. niger*, *Penicillium* sp. & *T. viride*) isolates (*E. coli*), yeast (*C. olivera*) and fungi (*Rhizopus* sp.).



**Fig. 3. Growth curves and parameters of tested fungal cultures grown on malt extract and Czapek's Dox media broth as well as *A. tubingensis* USMI03 RPf10 fermented solution as a whole medium or supplemented with sucrose during 8 days at 30°C using shake flasks as a batch culture**

a) Growth curves b) Growth parameters (Specific growth rate ( $h^{-1}$ ), multiplication rate (MR) and doubling time ( $t_d$ ))  
 ME= Malt extract medium, FSM= Fermented solution as whole medium, CD= Czapek's Dox medium, FSM+S= Fermented solution as whole medium +sucrose, C= control

#### 4. CONCLUSION

The fermented solution of *A. tubingensis* USMI03 RPF10 contains the highest concentration of citric acid, indole acetic acid (IAA) and elements as well as phosphatase activity. This solution was applied as mineral source for microbial growth. Using the fermented solution as mineral source (supplemented with sucrose) increased the growth of *Aspergillus niger*, *Penicillium* sp. and *Trichoderma viride*. about 1.94, 1.53 and 1.33-fold compared to that obtained by Czapek's Dox medium after 4 days incubation period at 30°C using shake flasks as a batch culture. It is suggested that, *A. tubingensis* USMI03 RPF10 as fermented solution can be used as a whole medium or as a mineral source for microbial growth but varied from one microorganism to another.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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