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# Anticancer and antioxidant activities of l- asparaginase produced by local *Weissella paramesenteroides* MN2C2 strain

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**Abstract**
Cancer is a major leading cause of death worldwide. However, lactic acid bacteria (LAB) were found to produce different anticancer agents, such as L-asparaginase (ASNase), which effectively contribute against the cancer cells growth. This study aimed for the production, partial purification, characterization and evaluation of both anticancer and antioxidant properties of ASNase from *Weissella paramesenteroides* MN2C2. The maximal ASNase production conditions were found to be reached when 10% (v/v) of the strain was inoculated in MRS medium of pH 7, then statically incubated for 48h at 37°C. The enzyme was precipitated by ammonium sulfate at 20-60% saturation giving 69.7 % recovery and 1.7 purification fold. The partially purified ASNase (PPASNase) showed molecular weight of 36 kDal, using SDS-PAGE investigation, as well as Km and Vmax of 4.41 mM and 130.72 U/ml/min respectively. The maximum activity of PPASNase was obtained at 35ºC and pH of 7.0 and was mostly retained at a wide range of temperature (20-60ºC) and pH (2-10). The anticancer effect of the PPASNase was tested against different cell lines. The most optimum $IC_{50}$ of 0.135 mg/ml was recorded against Caco-2 cells, while higher $IC_{50}$ values of 1.034, 4.5 and 1.636 mg/ml were obtained when tested against HepG-2, MCF-7 and A549 respectively. The PPASNase showed excellent antioxidant activity with an $IC_{50}$ of 235.8 µg/ml. The tested strain was also able to participate in the production of yoghurt with enhanced quality, which consequently comprises a number of health beneficial bioactive compounds. These results demonstrated that the probiotic *W. paramesenteroides* MN2C2 is a very promising microorganism for both pharmaceutical and nutritional uses.
**Keywords**: *Weissella paramesenteroides*,L-asparaginase, Anticancer activity, Antioxidant activity.

## 1. Introduction

L-asparaginase (ASNase: EC 3.5.1.1.) is an enzyme that catalyzes the breakdown of L-asparagine to L-asparticacid and ammonia [1]. Amongst the microbial ASNase sources, different microorganisms were studied such as *P. pseudoalcaligenes* JHS71 [2], *Bacillus sp.*[3] and *Staphylococcus* [4].

On the other hand, Lactic acid bacteria (LAB), which represent a beneficial group of microorganisms, have been broadly used in foodstuff fermentation worldwide since the metabolic effect of LAB was found to alter the nutritional property of food matrix producing a great number of substrates, and many bioactive end products, having especially beneficial effects on human health [5]. Therefore, LAB are considered as probiotics, which can sustain good health and significantly reduce the offset of many diseases [6]. Accordingly, fermentation of LAB was integrated in many applications related to the food industry [7].

One of the major products of LAB is the ASNase enzyme. The latter has attracted the attention of foodstuff processing manufacturers due to its ability to greatly lower the acrylamide production percentages, as ASNase can break L-asparagine which is a necessary precursor for acrylamide production [8]. Acrylamide is a carcinogenic substrate that is formed during food manufacturing as a result of the Maillard reaction [9].

Moreover, in several cancer cases, such as lymphoblastic leukemia, the anticancer activity of ASNase also results from the hydrolysis of L-asparagine, synthesized by normal cells, since the

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latter is imperatively needed by cancer cells in order to survive [10].

Although ASNase use was also proved to solve many other problems including neurotoxicity, hepatitis and many other diseases, unfortunately, some resulting dysfunctions were recorded which eventually caused some restrictions in its methodical applications and, consequently,the commercial uses of ASNase are still limited [11]. However, the ASNases enzymes produced by both *E. coli* and *Erwinia carotovora* were recently accepted for medical applications by the FDA.

Moreover, the oxidative effect of some substances plays very important roles in many human diseases such as cancer; atherosclerosis, emphysema, cirrhosis, and arthritis [12]. Consequently, antioxidants are very important to prevent cell damage that results from oxidative stress.

Beside the ability of *W. Paramesenteroides* MN2C2 to produce exopolysaccharide [13] [14], the locally isolated strain was also found to substantially decrease the pH of the cultivation medium due to the production of potent amounts of different acids, which is a common property of LAB [15].

Accordingly, in this study, the ASNase enzyme production by *W. Paramesenteroides* MN2C2 was optimized, partially purified, characterized and tested for its anticancer and antioxidant activities. The strain was also exploited for the possible production of dairy products, such as yoghurt, having enhanced nutritional values as well as advantageous medical uses.

## 2. Material and Methods

### *2.1. Microorganism and Culture conditions*

The strain was previously isolated, by the research team, from buffalo colostrums after 48 h of delivery, then identified as *Weissella paramesenteroides* MN2C2 and stored in the gene bank under the accession number of MK530206 [13]. The microorganism ($10^6$CFU/ml) was inoculated in De Man-Rogosa-Sharpe (MRS) broth medium (pH 6.5) and incubated aerobically at 37°C for 24 h in a static incubator. At the end of the incubation period, the culture was mixed to a solution of 40% glycerol and the resulting suspension was stored at -80$^o$C until needed [16].

### *2.2. Detection of the antitumor gene (ansA) by PCR*

To detect the *ans*A gene within the genomic DNA of *Weissella paramesenteroides* MN2C2, one set of PCR primers was designed according to the DNA sequence of *Lb. helveticus* (accession number: gi111610249), which was retrieved from the NCBI website. These primers included *ans*1F 5'ATGGAAAAGAAAAAGTTATTAT'3 and *ans*1R 5'AGCCACATCTGTATTGAAGAAATCT'3. PCR products were treated as described by Weisburg *et al*., (1991) [17]. The amplification program included the pre-denaturation of *ans*A gene at 95 ℃ for 5 min followed by 35 cycles each including denaturation at 94°C for 30 sec., annealing at 45°C for 30 sec., and extension at 72° C for 1 min (Biorad model ptc-1148).

### *2.3. Preparation of crude enzyme*

*Weissella paramesenteroides* MN2C2, of concentration 2% ($10^8$cfu/ml), was cultivated in 50 ml MRS broth supplemented with 10 g/L of L-asparagine and incubated at 37 °C for 48h. At the end of the incubation time, the culture was centrifuged at 10,000 rpm for 20 min at 4 °C. Finally, the supernatants, which represent the crude enzyme, were filter sterilized.

### *2.4. Measurement of ASNase activity*

ASNase activity determination was calculated using Nessler's methods [18] which rely on measuring the amount of ammonia release in order to estimate the rate of L-asparagine hydrolysis. The reaction mixture; which included 0.2 ml of 0.05 M Tris–HCl buffer, pH 8.6, 1.7 ml of 0.01M L-asparagine (Merck- Germany) and 100 µl bacterial culture supernatant; was incubated at 37 °C for 10 min. The reaction was then stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). The solution was centrifuged then 1 ml of Nessler's reagent was added to 500 µl of the supernatant and diluted with 7ml of $H_2O$. The absorbance of the colour which appearedafter 10 min, indicating the enzyme activity, was measured at A480 nm. Ammonium sulphate was used for standard curve preparation. Specific activity of ASNase was defined as the amount of µmoles of ammonia released per milligram (mg) protein per minute at 37°C [19]. The protein concentration in each sample was calculated according to the Bradford method using bovine serum albumin (BSA) as a standard [20].

### *2.5. Effect of different cultivation factors on ASNase production (incubation temperature, pH, time and inoculum size)*

To determine the maximum ASNase production from *Weissella paramesenteroides* MN2C2, an inoculum size of 8% was used to cultivate the tested microorganism in MRS medium adjusted at pH 6.5 for 24 h at different incubation temperatures, including 25, 30, 35, 40 and 45°C. The optimum initial pH of the medium was also studied by its adjustment to a range of pH of 5 – 8 using either 0.1 M NaOH or 0.1 M HCL. Moreover, different incubation times that ranged from 6 to 60 h, were studied as well. Finally, different inocula sizes, ranging from 2-12 % were also tested.

### *2.6. Partial purification of ASNase*

Ammonium sulfate salt was added under cooling and stirring conditions to the concentrated crude enzyme solution to a final concentration of 20% then the precipitate was centrifuged and removed. Thereafter,

the ammonium sulfate concentration was increased up to 60% and the second precipitate was obtained after centrifugation at 10,000 rpm for 20 minutes. The final precipitate was resuspended in a minimal volume of 0.05 M Tris-HCl of pH 7.5 and dialyzed. Finally the enzyme activity and protein concentration of the dialyzed solution was quantitatively assayed using Nessler's and Bradford methods [18,20].Then the partially purified ASNase (PPASNase) was stored at 4°C [21].

### *2.7. Characterization of PPASNase*

#### *2.7.1. Molecular weight determination of PPASNase*

The molecular weight (MW) of PPASNase was determined according to the method of Laemmli, (1970) [22] using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).The concentration of SDS used to prepare the separation polyacrylamide gel was 15% (w/v) while that used for the preparation of the stacking gel was 5% (w/v). Electrophoresis was performed at 100V. The PPASNase was stained with Coomassie Blue then, the gel was transferred to a de-staining solution. Finally, a gel documentation system was used for imaging the resulting stained proteins which appeared as visible blue bands [23]. The obtained molecular weights of the resulting bands were detected based on those of the coinciding bands on the standard protein ladder and compared to the expected MW calculated based on the number of base pairs, or the number of amino acids, reported, for ansA gene of the 100% similar strain, on the NCBI website.

#### **2.7.2** *Factors affecting enzyme activity*

#### *2.7.2.1 Enzyme concentration*

Optimum PPASNase concentration was assessed by using different concentrations of the enzyme, ranging from 0.1 to 2.1 mg in the reaction mixture.

#### *2.7.2.2. Kinetic parameters*

The value of Michaelis constant (Km) as well as that of the maximum velocity (Vmax) were determined based on Lineweaver-Burk plot using L-asparagine as substrate in different concentrations ranging from 1.5 to 224 mg/ml.

#### *2.7.2.3. Effect of temperature on enzyme activity and stability*

In order to determine the optimal temperature for enzyme activity, the reaction mixture was incubated at different temperatures ranging from 20 to 90°C. The thermal stability of PPASNase was further tested by pre-incubating the PPASNase at the same range of temperature for 30 minutes, after which the residual enzyme activity was determined using the above mentioned activity assay method.

#### *2.7.2.4. Effect of pH on enzyme activity and stability*

The effect of pH was assessed using 3 different molarities of Tris-HCl buffer (pH from 2 to 11). The pH stability of PPASNase was also examined by pre-subjecting the enzyme solution to the same range of pH values for 30 minutes. At the end of each incubation period, the activity of the pretreated PPASNase enzyme was determined using the activity assay method.

### *2.8. In vitro anticancer activity*

The cytotoxicity of PPASNase was evaluated at the Faculty of Agriculture; Cairo University Research Park (CURP), Embryology and Cell Culture Lab. Cell lines of different cancer types were tested including cells of breast cancer (MCF-7), colorectal adenocarcinoma (Caco-2), hepatocellular carcinoma (HepG-2) and lung cancer (A549) then compared to normal human lung cells (Wi-38). Each test was performed by inoculating a 96-well microtiter plate containing Roswell Park Memorial Institute (RPMI 1640) medium with a concentration of $10^3$ cell/ well. The plats were then incubated at 37$^o$Cfor 24 h in a 5% CO2 incubator. Fresh, serum free medium was then added followed by the addition of different concentrations of the PPASNase samples ranging from 0.25-1 mgml$^{-1}$. After that, the plates were further incubated for another 48 h for cells activation. Negative control plates, containing cells without PPASNase, were also prepared and incubated. Moreover, positive control plates, containing 50 µg ml$^{-1}$ of Doxorubicin standard to guarantee 100% lethality, were prepared as well.

To the activated cells in each well, 40 µl of 2.5 µg ml$^{-1}$ MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-Diphenly Tetrazolium Bromide) were added and the plates were reincubated at 37$^o$C for 4 h. The reaction was stopped by the addition of 200 µl of 10% SDS to each well followed by overnight incubation. Finally, the absorbance of each resulting solution was evaluated at 540 nm using a MicroplateMulti-Well Reader [24].

Viability % = [(Reading of extract/Reading of negative control) – 1] × 100

### *2.9. Selectivity index (SI)*

The selectivity index values were calculated according to the following equation:

SI = IC50 of normal cells/IC50 of cancer cells.

High SI values (> 2) indicate significant selective toxicity towards cancer cells, however low SI values

(< 2) reveal that the tested compounds are broadly toxic to normal cells [25].

### *2.10. Antioxidant activity*

The antioxidant activity of PPASNase was determined using the Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay method [26]. Therefore, 100 µl of different concentrations of PPASNase including 40, 80, 120,160, 200, 240 µg/mL, were dissolved in methanol and mixed to 900 µl of DPPH and incubated in a dark place for 30 min. The results of the absorbance reduction, caused by the scavenging of the DPPH free radicals by PPASNase, were determined at 517 nm and compared to that of control samples containing the same concentration of DPPH in methanol. The % free radical scavenging was then calculated according to the following equation:

Free radical scavenging % = [(X control-Ẍ sample)/X control] ×100.

Where Ẍsample represents the absorbance of DPPH solution with sample and X control represents that of DPPH solution in methanol without sample.

The IC50 value, which represent the concentration of PPASNase required to scavenge 50% of the DPPH free radicals, was also evaluated.

### *2.11. Application of probiotic Weissella paramesenteroides***MN2C2**

#### *2.11.1. Milk*

Fresh buffalo's milk, used for the manufacture of yoghurt, was obtained from the Faculty of Agriculture, Cairo University. The approximate chemical composition of this milk was (g/100 g): 4.00 protein, 7.00 fat, 0.80 ash, 17.00 total solids, 5.00 lactose as well as 82 % water.

#### *2.11.2. Tested probiotic strain starter culture*

The probiotic strain, used to ferment the buffalo's milk, was previously isolated by the research team from buffalo colostrums after 48 h of delivery and identified as *Weissella paramesenteroides* MN2C2 then stored in the gene bank under the accession number of MK530206 [13] [14].

#### *2.11.3. Control probiotic strains starter culture*

Control probiotic yoghurt fermentation culture (ABT), commonly used by yoghurt manufacturing industries in Egypt, was acquired from the Dairy sciences department, Faculty of Agriculture, Cairo University, Egypt (Chr. Hansen's, Denmark). The ABT culture contains inocula of *Lactobacillus acidophilus*, *Bifidobacteriumbifidum* and *Streptococcus thermophilus*.

#### *2.11.4. Preparation of yoghurt*

Fresh buffalo's milk was subjected to heat treatment at 85°C for 10 min, then left to cool at 42°C [27],then transferred into 100 ml cups and categorized as C, T1 and T2. First of all, category C cups, which represented the control samples, were inoculated with 2.5% of ABT yoghurt culture.On the other hand, category T1 cups were inoculated with different concentrations of probiotic *W. paramesenteroides* MN2C2 ranging from 5 to 20%. Finally, category T2 cups were inoculated with a mixture of 2.5% of ABT yoghurt culture and 15% of probiotic *W. paramesenteroides* MN2C2. The inoculated milk samples were incubated at 40°C for 4 h then refrigerated at 4 ± 1°C.

#### *2.11.5. Yoghurt evaluation*

##### *2.11.5.1. Quality evaluation*

The quality of the obtained yoghurt samples was evaluated through the measurement of the total viable count (TVC) of bacteria in each samples as well as the estimation of both the resulting pH and the acidity of these samples. The TVC of the treated samples was performed by serially diluting 1 ml from each samplein saline solution (0.9%) then spreading each appropriate dilution onto triplicates of MRS agar plates, which were incubated for 48 h at 37 °C. After the incubation period, the colonies formed on each plate were counted. The pH value of each sample was also measured using a digital laboratory Jenway 3510 pH meter, UK. Finally, titratable acidity (TA) was also measured according to AOAC method [28].

##### *2.11.5.2. Sensory evaluation*

Fresh samples of yoghurt were sensory evaluated by 50 members of both the Chemistry of Natural and Microbial Products and those of the Dairy science Departments, National Research Centre, Cairo, Egypt. The yoghurt was graded for its taste and flavor (out of 60 points), its body and texture (out of 30 points), and its color and appearance (out of 10 points) according to the score card method suggested by Keating and White 1990 [29].

### *2.12. Statistical analysis*

All of the determinations reported in this study were performed in triplicate, and the results are presented as mean values.

## *3. Results and discussion*

### *3.1. Detection of ASNaseans Agenes*

The results shown in Fig. 1 prove that *Weissella paramesenteroides* MN2C2 strain possesses *ans*A gene with an amplicon in the same size predicted according to the preliminary bioinformatics analysis of the partial gene sequencing. This analysis also

showed that *ans*A gene operon from the tested strain was 100% similar to *Weissella paramesenteroides* strain FDAARGOS (accession number CP023501.1). Moreover, the strain was also found to have lower percentages of similarities, of 84.8, 80.7 and 70.3%, with different previously identified strains such as *Weissella jogaejeotgali* strain FOL01 (accession number CP014332.1), *Weissella hellenica* strain CBA3632 (accession number CP042399.1) and *Weissella confusa* strain VTT E-90392 (accession number CP027565.1) respectively.

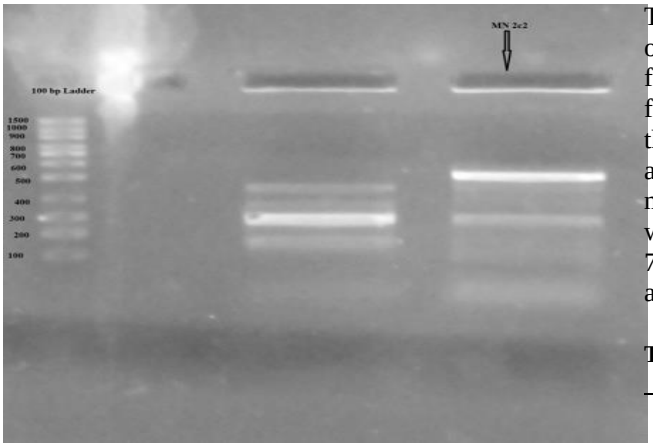


**Fig. 1. PCR product profile of ansA gene extracted from the genomic DNA of *W.paramesenteroides*MN2C2.**

### *3.2. Optimization of ASNase production (incubation temperature, pH, time and inoculum size)*

The results illustrated in Fig. 2A revealed a direct correlation between the incubation temperature and the number of CFU, as well as the activity of the produced ASNase, up to 37°C after which a notable decline in both parameters was detected. Likewise, when the initial pH of the tested fermentation medium was studied, the results showed that the number of CFU, and consequently the activity of the ASNase enzyme, improved as the initial pH of the medium was shifted towards neutral pH up to 7 after which a significant decrease in the number of CFU, accompanied by a gradual decrease in the enzyme activity was noticed (Fig 2B). Moreover, the results also showed that the maximum number of CFU as well as the maximum enzyme activity was reached when the incubation time was extended up to 48 h (Fig. 2C). On the other hand, Fig 2D revealed no significant difference between the number of CFU or the ASNase activity detected upon using an inoculum size of 10 or 12% indicating that the inoculum size of 10% was therefore much favored.

The activity of ASNase produced from different microbial sources was found to depend on the incubation temperature and the pH of the fermentation medium [30]. Maximum enzyme activity conditions recorded in this study were similar to those reported by Basha *et al*., (2009) [31] who studied the production of ASNase by some marine actinomycetes strains. The obtained data were more or less in agreement with those of Alrumman *et al*., (2019) [23] who reported that the maximal ASNase production by *B. licheniformis* KKU-KH14, was achieved using an optimum pH of 6.5 and an incubation temperature of 37 °C.

### *3.3. Partial purification of* ASNase

The specific activity of crude ASNase extract and that of its partially purified form (PPASNase), obtained from the dialyzed 60% ammonium sulphate treated fraction, were calculated (Table 1). The results showed that, this step led to a 1.7 fold increase in purification along with recovery a yield of 69.7%. These results more or less agreed with those of many investigators who reported a recovery yield ranging between 61 to 77% [32,33,34,35,36] and a purification fold of 1.44 and 1.5[33,36].

**Table 1. Precipitation of ASNase by ammonium sulfate**

| Purification Steps | Enzyme activity (U) | Protein concentration (mg/ml) | Specific activity (U/mg protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude ASNase | 310 | 7.3 | 42.5 | 1.00 | 100 |
| PPASNase | 216 | 3.0 | 72 | 1.70 | 69.7 |

### *3.4. Molecular weight identification of PPASNase by SDS-PAGE*

The PPASNase was examined using SDS-PAGE technique in order to determine its molecular weight (Fig. 3). The result showed 4 distinctable protein bands. However, the protein band which approximately coincided to that of the standard protein ladder of molecular weight 36 kDa most probably represented that of the partially purified protein as this MW was very close to that predicted by the calculations based on the information retrieved from the NCBI website, including the number of ansA gene base pairs (990 bp, accession number NZ_CP023501 ) as well as that of the amino acids components of the ansA protein (329 aa, accession number WP_040760604) of *Weissella paramesenteroides* strain FDAARGOS previously proved to be a 100% similar strain (accession number CP023501.1) . These data were very similar to those of Radha *et al*., (2018) [37] who reported that the molecular weight of the purified ASNase produced by *V. cholerae*, which gene was a recombinant obtained by it's over expression in *E. coli*, was 36.6 kDa

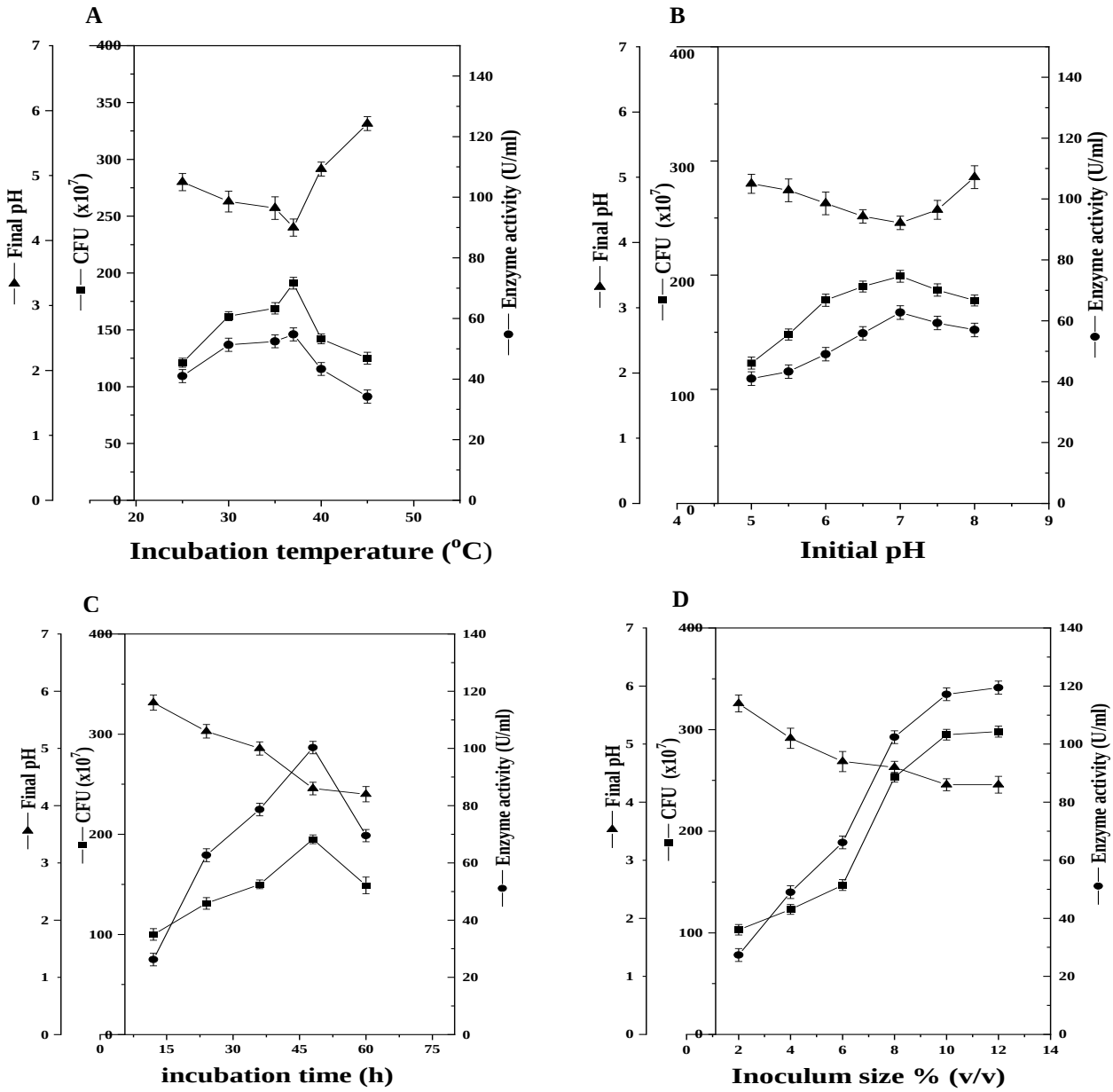


**Fig. 2. Optimization of ASNase production by *Weissellaparamesenteroides* MN2C2: A. Incubation temperature, B. Initial pH, C. Incubation time, and D. Inoculum size.**

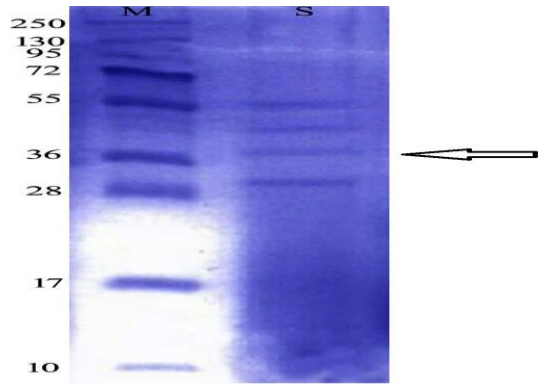


**Fig. 3. Molecular weight determination of PPASNase using SDS-PAGE technique.**

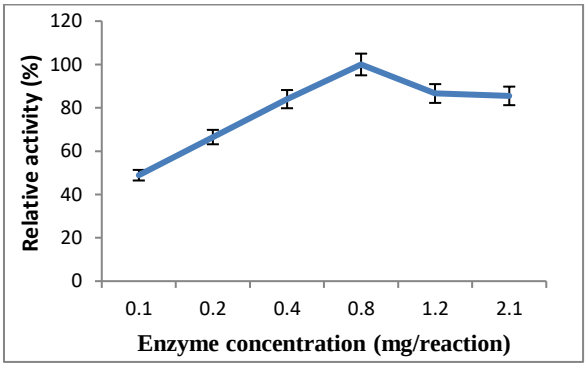


**Fig. 4. Effect of different enzyme concentrations on the PPASNase activity**

### *3.5. Characterization of PPASNase*

#### *3.5.1. Optimum enzyme concentration*

The results in figure 5 reveal that the optimum enzyme concentration of 0.8 mg/reaction was able to liberate maximum amounts of $NH_3$ from L-asparagine solution of 0.01 M revealing the high activity of the produced enzyme. This is in accordance with Basha *et al*., (2009) [31].

#### *3.5.2. Kinetic parameters*

Km is defined as the concentration of substrate which permits the enzyme to achieve half Vmax. In this study, PPASNase was found to have low Km and high Vmax indicating a high affinity for the substrate and consequently, V max is achieved using a low substrate concentration [38]. Although a km of 4.7 mM was reported by Han *et al.*, (2015) [39] for L-asparaginase produced by *Bacillus coagulance*, the Km values calculated by many other investigators were much higher [40]. Moreover, the results of the obtained V max were found to exceed those previously reported by Han *et al.*, (2015) [39] and Basha *et al.*, (2009) [31].

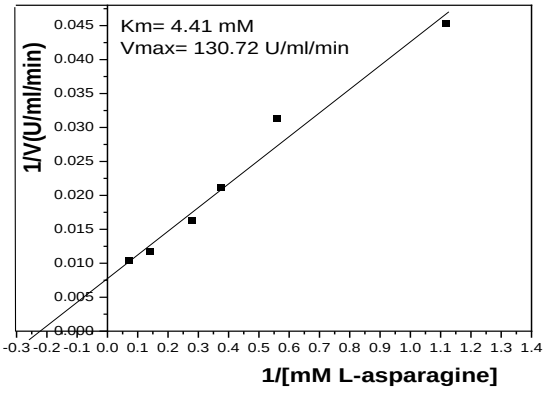


**Fig. 5. Determination of the kinetic parameters Km and Vmax applying Lineweaver-Burk plot**

#### *3.5.3. Effect of temperature on enzyme activity and stability*

Testing of the temperature activity (Fig. 6A) and stability (Fig. 6B) of the PPASNase reveal that although it's maximum activity was recorded at 35°C, the enzyme had a great ability to sustain a very wide range of temperature that extended from relatively warm temperatures of 25 to 30°C to as hot as 60°C with a narrow window of relative activity variation that ranged from 100 to 60%. The enzyme was also found to resist heat denaturation and still be active beyond this temperature since more than 35% of the initial activity were still detected as the temperature was raised up to 90°C, indicating that it is a thermostable enzyme.

These data are in agreement and even more or less surpass those of Desai and Hungund (2018) [41] as well as those of Muneer *et al*., (2020) [30]. The former reported that the optimum temperature for L-asparaginase from *Streptomyces sp.* isolated from soil was 35°C, and was able to retain its activity up to 50°C, and the later proved its activity between 37 and 85 °C. However, the optimum temperature of L-asparaginase produced from some hyperthermophilic microorganism was found to be between 85 and 100°C [42].

#### *3.5.4. Effect of pH on enzyme activity and stability*

The same trend of results was also noticed upon studying the pH activity (Fig. 7A) and stability (Fig. 7B) of the produced PPASNase since although the maximum activity of the enzyme was detected in the neutral pH range of 7 to 7.5 which is approximately the pH of human blood,, substantial percentages of enzyme activity of more than 50% was still evident in both extreme acidic and alkaline pH of 2 and 11 respectively.

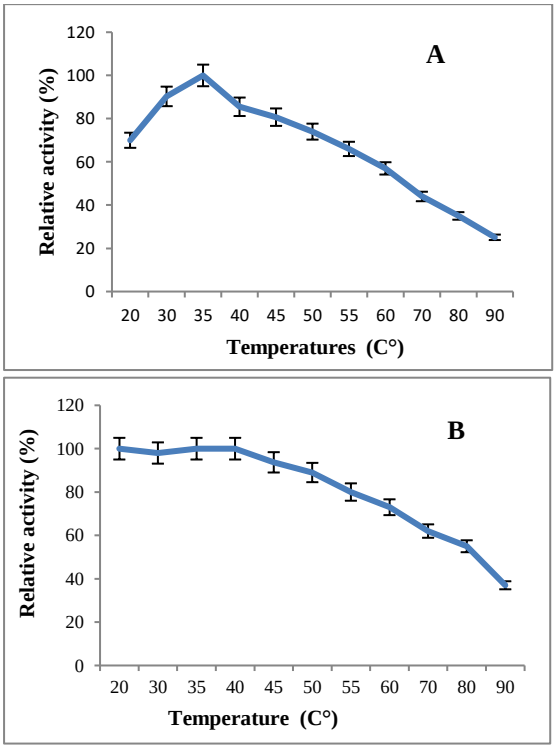


**Fig. 6. A) Effect of different temperatures on the PPASNase activity and B) stability**

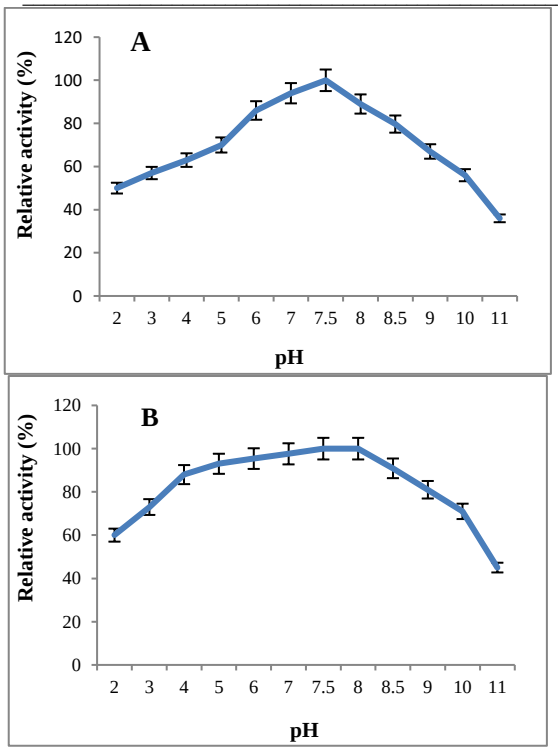


**Fig. 7. A) Effect of different pH values on the PPASNase activity and B) stability**

These results could be attributed to the probiotic nature of the producing microorganism that was previously proved by our research team to sustain highly extreme acidic pH of the stomach, which could reach 1.5, as well as the high alkalinity of the intestinal part of the digestive system, especially that of the large intestine [14]. Almost all L-asparaginases, isolated from microbial sources, were only found to be stable in the pH range of 6.0 to 10 [43], however, to our knowledge, no previous study reported the stability of L-asparaginase enzyme under both of these two extreme pH conditions.

### *3.6. Anticancer activity of PPASNase*

The results of the anticancer activity of PPASNase enzyme revealed that the lowest obtained IC50 value of 0.135mg/ml, and consequently the highest SI value of 2.23, was obtained when the enzyme was tested against Caco-2 cell line. This result proved the enhanced effect of the PPASNase enzyme against colon cancer cells which represent a very lethal type of cancer that causes a high percentage of mortality every year worldwide [44]. Moreover, according to Awang *et al*., (2014) [25], the obtained SI value is considered very safe for human treatment.
Unfortunately, much higher IC50 values of 1.636 mg/mland 1.034 mg/mlwere obtained when the enzyme was tested against both A549 and HepG-2 respectively. These IC50 corresponded to SI values of 0.18 and 0.29 respectively. Also, testing of the enzyme against MCF-7 resulted in a considerably high IC50 of 4.5 mg/ml and an insignificant SI value of 0.067.

**Table 2. Anticancer activity of PPASNase**

| Cell Line | PPASNase | |
|---|---|---|
| | $IC_{50}$ (mg/ml) | SI |
| **Caco-2** | 0.135 | 2.23 |
| **HepG-2** | 1.034 | 0.29 |
| **MCF-7** | 4.500 | 0.067 |
| **A549** | 1.636 | 0.18 |
| **Wi-38** | 0.301 | 1.00 |

### *3.7. Antioxidant activity of PPASNase*

The results in Table 5 showed that the scavenging activity of 50.89% was recorded when the PPASNase sample concentration of 240 µg/ml was used. Therefore, the concentration of PPASNase required to scavenge 50% of the DPPH free radicals (IC50) was calculated as 235.80µg/ml.

This recorded IC50 result was found to be much lower than those reported by many investigators such as Maysa *et al*., (2010) [45] and Rani *et al*., (2011) [46] who reported higher IC50 of 325.4 and 263.63 µg/ml for PPASNase produced by *Bacillus* sp R36 and *Aspergillusflavus* (KUFS20) respectively, thus revealing the high antioxidant activity of the tested PPASNase.

**Table 3 . Antioxidant activity of PPASNase**

| PPASNase Sample conc. (µg) | Free DPPH radical scavenging (%) | IC50 (µg/ml) |
|---|---|---|
| 40 | 38.24 | 235.80 |
| 80 | 39.39 | |
| 120 | 40.70 | |
| 160 | 43.51 | |
| 200 | 46.91 | |
| 240 | 50.89 | |

### *3.8. Yoghurt quality evaluation*

The results in Fig. 4 revealed that the TVC of all samples increased with the storage period which extended for15 days. The TVC recorded in the fresh samples of categories C, T1 and T2 were 80, 65 and 149 CFU $X10^7$ respectively. These results increased gradually reaching 249, 191 and 294 CFU $x10^7$ respectively at the end of the storage period. These results were in agreement with fayed *et al.*, (2019) [27] and Ismail *et al*.,(2020) [47], who indicated that the viable counts of probiotic strains and starter cultures enhanced during storage periods.

The microbial counts in all samples also correlated with the increase in the acidity and pH values. However, after 15 days of storage, the acidity value of T2 of 1.3 was found to be higher than those of C and T1, which were recorded as 1.16 and 1.12 respectively. On the other hand, changes in pH values of all experimental samples were also recorded for both fresh and stored samples. The results of the pH of fresh samples of categories C, T1 and T2 were 4.7, 4.9 and 4.6 respectively. These values significantly dropped during the storage period to reach 4.4 for samples of categories C and T1 and 4.2, for those of category T2. These results were confirmed by Assem, *et al.*, (2019) [48] who found that the acidity increased during storage time. Also, El-Shafei *et al.*, (2018) [49] indicated that the pH values in all samples decreased through the storage period.

### *3.9. Sensory evaluation*

The sensory properties reflect the quality and shelf-life of dairy products. According to the results presented in table 4, the fresh T2 samples were granted the highest total score of 94% for sensory evaluation as it was found to surpass both fresh C and T1 samples in their taste and flavors as well as in their color and appearance. T2 samples were also much better in their body and texture than T1 samples.

**Table 4. Sensory evaluation of fresh yoghurt at 5 ± 1ºC**

| Samples category | Taste and Flavor (out of 40 points) | Body and texture (out of 40 points) | Color and appearance (out of 20 points) | Total (out of 100 points) |
|---|---|---|---|---|
| C | 37±0.2 | 38±0.3 | 17±0.2 | 92±0.2 |
| T1 | 34±0.2 | 33±0.2 | 14±0.2 | 81±0.2 |
| T2 | 38±0.3 | 38±0.3 | 18±0.3 | 94±0.3 |

## *Conclusion*

The results of this study showed that, *W. paramesenteroides* MN2C2 was able to produce ASNase with anticancer and antioxidant properties. The maximum rate of ASNase production was studied and was found to depend on different interactions between time, pH and temperature. The PPASNase enzyme was also proved to be able to sustain both acidic and alkaline extreme pH values as well as a very wide range of temperature variation. Therefore, according to this study the potential use of this productas an anticancer or antioxidant drug should be emphasized. However, more reports are still needed in order to assess this possibility, such as *in vivo* studies and some pharmacodynamic profile studies on animal models as well as many human clinical trials. Moreover, the study also proved the ability of *W. paramesenteroides*to successfully convert milk into yoghurt. However the result of the sensory evaluation test recommended the mixing of the probiotic strain with traditional yoghurt starter cultures in order to produce a dairy product with enhanced sensory properties, as well as, possessing many prophylaxis and therapeutic effects.

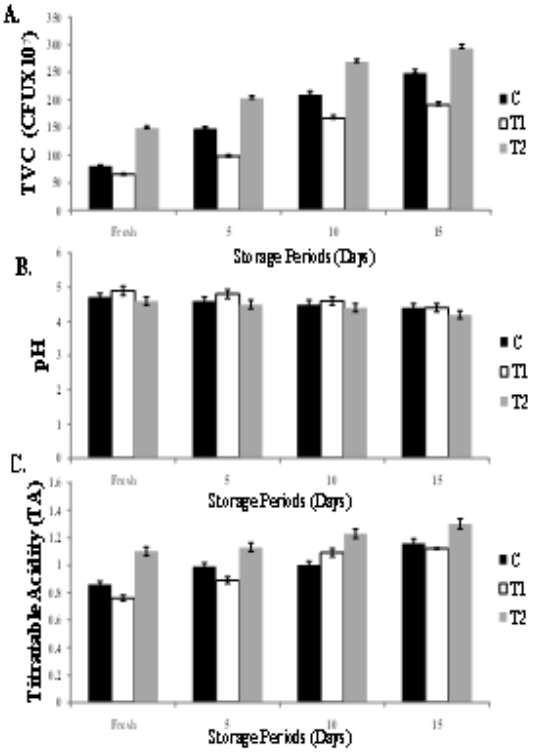


**Fig. 8. Effect of different storage period of yoghurt samples on: A. TVC, B. pH, and C. Acidity**

### *Declaration of competing interest*

The authors declare that is no conflict of interest.

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### *Authors' contributions*

Mai Amer contributed to conceptualization, formal analysis, funding acquisition, methodology, resources, and writing the original draft. Nagwa Atwa and Eman Elgammal contributed to conceptualization, formal analysis, supervision, writing the original draft, review, and final editing. Ahmed Eldiwany, Ferial Rashad and Insaf Dawoud, contributed to conceptualization, formal analysis, and supervision.

### *References*

[1] Shabana, A.M.I., Shetaia, Y.M., Abdelwahed, N.A.M., Esawy, M.A., Alfarouk, O.R. Optimization, purification and antitumor activity

of kodamaeaohmerimri anomy L-asparaginase isolated from banana peel. *Curr. Pharm. Biotechnol.*, 22(5):646–663(2021).

[2] Badoei-Dalfard, A., L-asparaginase production in the *pseudomonaspseudoalcaligenes* strain JHS-71 isolated from Jooshan Hot-spring. *MolBiol Res Commun*., 5(1):1-10(2016).

[3] Vidhya, M.,Aishwarya, R.,Alagarsamy, S. Production, purification and characterization of extracellular L-asparaginase from a soil isolate of Bacillus sp. *African Journal of Microbiology Research*, 4: 1862-1867(2010).

[4] Han, S., Jung, J. and Park, W., Biochemical characterization of L-asparaginase in NaCl tolerant *Staphylococcus* sp. OJ82 isolated from fermented seafood. *Journal of Microbiology and Biotechnology* 24: 1096-1104(2014).

[5] Zielińska, D. and Kołożyn-Krajewska, D., Review Aritcle; Food-origin lactic acid bacteria may exhibit probiotic properties. *Bio. Med. Research International*, 159(2018).

[6] Markowiak, P. and Śliżewska, K., Review: Effects of probiotics, prebiotics, and symbiotics on human health. *Nutrients*, 9: 1021(2017).

[7] Sanilier, N.,Gokcen, B.B. and Sezgin, A.C., Health benefits of fermented foods. *Critical Reviews in Food Science and Nutrition*, 59: 506-527(2019).

[8] Thomas, M.A.,Schobachler, B., Escher, F. Acrylamide in gingerbread: critical factors for formation and possible ways for reduction. *Journal of Agricultural and Food Chemistry*, 51: 4282-4288(2014).

[9] Fernanda, F.,Gonçalves, D.,Stanislau, B. J. Acrylamide mitigation in French fries using native L-asparaginase from *Aspergillusoryzae*CCT 3940. *Food Science and Technology*, 222-229 (2017).

[10] Lopes, A.M. *et al.*, Therapeutic L-asparaginase: upstream, downstream and beyond.*Crit. Rev. Biotech.*, 37, 82–99 (2017).

[11] Jamie, K., James, M. and Stefan, F., Safety, efficacy, and clinical utility of L-asparaginase in the treatment of adult patients with acute lymphoblastic leukemia. Onco. *Targets and Therapy*, 10: 1413-1422(2017).

[12] Nithiya, S.,Karthik, N. and Jayabharathi, J. *In vitro* antioxidant activity of hundred piperidone derivatives. *Intl. J. Pharm. Pharm. Sci.*, 3: 254-256,(2011).

[13] Amer, M.N., Elgammal, E.W., Atwa, N.A., Eldiwany, A.I., Dawoud, I.E. and Rashad, F.M., Structure elucidation and in vitro biological evaluation of sulfated exopolysaccharide from LAB *Weissellaparamesenteroides* MN2C2. *Journal of Applied Pharmaceutical Science*, 11 (05):022-031(2021) (a).

[14] Amer, M.N.,Eldiwany, A.I., Elgammal, E.W., Atwa, N.A., Dawoud, I.E. and Rashad, F.M,. Nano-Exopolysaccharide from the Probiotic Weissella paramesenteroides MN2C2: Production, Characterization and Anticancer Activity. *Egypt. J. Chem.* 64(12):7023 – 7033 (2021) (b).

[15] Mora-Villalobos, J.A., Montero-Zamora, J.,Barboza, N., Rojas-Garbanzo, C.,Usaga, J., Redondo-Solano, M.,Schroedter, L.,Olszewska-Widdrat, A. andLópez-Gómez, J.P., Multi-Product Lactic Acid Bacteria Fermentations: A Review. *Fermentation* , *6*, 23 (2020).

[16] Lavanya, B., Sowmiya, S., Balaji, S. and Muthuvelan, B., Screening and characterization of lactic acid bacteria from fermented milk. *Br. J. Dairy Sci.*, 2(1): 5-10 (2011).

[17] Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J., 16S ribosomal DNA amplification for phylogenetic study. *J.Bacteriol.* 173:697–703(1991).

[18] Worthington Biochemical Corporation, Available from, *Worthington Enzyme Manual* (1993).

[19] Kafkewitz, D. and Goodman, D., L-asparaginase production by the rumen anaerobe *Vibrio succinogenes*. *J. Appl. Microbiol.*,27:206–209 (1974).

[20] Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteinedye binding. *Anal. Biochem.*,72:248-254 (1976).

[21] Dhevagi, P. and Poorani, E., Isolation and characterization of L-asparaginase from marine actinomycetes. Ind. J. Biotech., 5:514-520 (2005).

[22] Laemmli, U., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227, 680–685 (1970).

[23] Alrumman, S.A., Mostafa, Y.S., Al-izran, K.A., Alfaifi, M.Y., Taha, T.H. and Elbehairi, S.E., Production and Anticancer Activity of an L-asparaginase from *Bacillus licheniformis* Isolated from the Red Sea, Saudi Arabia. *Scientific reports*, 9:3756 (2019).

[24] Thabrew, M.I., Hughes, R.D. and McFarlane, I.G., Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. *J. Pharm. Pharmacol.*, 49, 1132–1135 (1997).

[25] Awang, N., Aziz, Z.A., Kamaludin, N.F. and Chan, K.M., Cytotoxicity and mode of cell death induced by Triphenyltin (IV) compounds *in vitro*. *J. Biol. Sci.*, 14, 84-93(2014).

[26] Yin, J.Y., Nie, S.P., Zhou, C., Wan, Y. and Xie, M.Y., Chemical characteristics and antioxidant activities of polysaccharide purified from the seeds of Plantago. Asiatica. *L. J. Sci. Food Agric.*, 90, 210–217(2010).

[27] Fayed, B., El- Sayed, H.S., Abood, A., Hashem, A.M., andMehanna, N.S., The application of multi- particulate microcapsule containing probiotic bacteria and inulin nanoparticles in enhancing the probiotic survivability in yoghurt.*Biocatalysis and Agricultural Biotechnology*, 22, 101391(2019).

[28] AOAC., Official Methods.Agricultural Chemicals, Contaminants, Drugs, 19thWashington, DC, USA: *AOAC International*(2012).

[29] Keating, K.R. and White, C.H. Effect of alternative sweeteners in plain and fruit-flavored yogurts. *J. Dairy Sci.*, 73: 54-62(1990).

[30] Muneer, F., Siddique, M.H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., Afzal M. and Nadeem, H. Microbial L-asparaginase: purification, characterization and applications. *Archives of Microbiology*,(2020).

[31] Basha, N.S., Rekha, R., Komala, M. and Ruby, S. Production of Extracellular Anti-leukaemic Enzyme Lasparaginase from Marine Actinomycetes by Solidstate and Submerged Fermentation: Purification and Characterisation. *Trop. J. Pharm. Res.*, 8 (4): 353(2009).

[32] Muslim, S.N. Production, purifcation and characterization of a novel L-asparaginase from *Acinetobacter baumannii* with anticancerous activity. *Int. J. Curr. Eng. Technol*., 4(1):6–11(2014).

[33] Mishra, A. Production of L-asparaginase, an anticancer agent, from *Aspergillus niger* using agricultural waste in solid state fermentation. *Appl. Biochem. Biotechnol*.,135:33–42(2006).https://doi. org/10.1385/ABAB:135:1:33.

[34] Singh, Y., Gundampati, R.K., Jagannadham, M.V. and Srivastava, S.K. Extracellular L-asparaginase from a protease-defcient *Bacillus aryabhattai* ITBHU02: purifcation, biochemical characterization, and evaluation of antineoplastic activity in vitro. *Appl. Biochem. Biotechnol*., 171:1759–1774(2013).https://doi.org/10.1007/s1201 0-013-0455-0.

[35] Zhang, S., Xie, Y., Zhang, C., Bie, X., Zhao, H., Lu, F. and Lu, Z. Biochemical characterization of a novel L-asparaginase from *Bacillus megaterium* H-1 and its application in french fries. *Food Res. Int*., 77:527–533(2015). https://doi.org/10.1016/j.foodres.2015.08.031.

[36] Qeshmi, F.I., Homaei, A., Fernandes, P. and Javadpoure, S. Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. *Microbiol. Res* .,208:99–112(2018). https://doi.org/10.1016/j.micres.2018.01.011.

[37] Radha, R., Arumugam, N. and Gummadi, S.N.,Glutaminase free L-asparaginase from Vibrio cholerae: Heterologous expression, purification and biochemical characterization.Int. J. Bio. Mac. 111, 129–138(2018).

[38] Javed, S., Azeem, F., Hussain, S., Rasu, I., Siddique, M.H., Riaz, M., Afza, M., Kouser, A. and Nadeem, H. Bacterial lipases: a review on purifcation and characterization. *Prog. Biophys. Mol. Biol.*, 132:23–34(2018).

[39] Han, S., Jung, J. and Park, W. Biochemical characterization of L-asparaginase in NaCl-tolerant *Staphylococcus sp*. OJ82 isolated from fermented seafood. *J. Microbiol. Biotechnol.*, 24:1096–1104(2015).

[40] Chohan, S.M. and Rashid, N. TK1656, a thermostable l-L from *Thermococcus kodakaraensis*, exhibiting highest ever reported enzyme activity. *J. Biosci. Bioeng.* 116:438–443(2013).

[41] Desai, S.S. and Hungund, B.S. Submerged fermentation, purification, and characterization of L-asparaginase from *Streptomyces sp.* isolated from soil. *J. Appl. Biol. Biotechnol*., 6, 17–23 (2018).

[42] Jia, R., Wan, X., Geng, X., Xue, D., Xie, Z. and Chen, C. Microbial L-asparaginase for Application in Acrylamide Mitigation from Food: Current Research Status and Future Perspectives. *Microorganisms*., 9(8):1659 (2021).

[43] Ghasemi, A., Asad, S. and Kabiri, M. Cloning and characterization of *Halomonas elongata* L-asparaginase, a promising chemotherapeutic agent. *Appl. Microbiol. Biotechnol*. 101, 7227–7238 (2017).

[44] Sung, H., Ferlay, J., Siegel, R.L.,Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F., Global cancer statistics 2020: GLOBOCAN estimates of incidence andmortality worldwide for 36 cancers in 185 countries, CA, *A Cancer J. Clin*.,(2021).

[45] Maysa, E.M., Amira, M.G.E., Sanaa, T. and Sayed, E.I., Production immobilization and antitumour activity of L-aspraginase of *Bacillus sp* R36. *Journal of American Science*, 6 (8):157-165(2010).

[46] Rani, S.A., Sundaram, L. and Vasantha, P.B.,In vitro antioxidant and anticancer activity of L-asparaginase from *aspergillus flavus*(kufs20). *Asian J. Pharm. Clin. Res*, 4:l 2, 174-177 (2011).

[47] Ismail, E., Shenana, M., Elalfy, M., Essawy, E. and Abdelhahim, S., Novel Probiotic Adjunct Cultures for the Production of Fruit-Flavoured Drinkable Yoghurt. *Egyptian Journal of Food Science*, 48(2), 213-228 (2020).

[48] Assem, F., shafae, K., Samir, H., Matter, M., Hanafy, M. and Amer, A., Effects of Carnation Essential Oil Extracted from *Carnation Calli* on Extending Shelf Life of Yoghurt. *Plant Tissue Cult. & Biotech*. 29 (1): 1-14 (2019).

[49] El-Shafei, K., Elshaghabee, F.M.F., El-Sayed, H.S. and Kassem, J.M., Assessment the viabil-ity properties of *Lactobacillus casei* strain using Labneh as a carrier. *Acta. Sci. Pol.Technol. Aliment*. 17 (3), 267–276 (2018).