

**Aim of the Experiment:****DETERMINATION OF ANTIOXIDANT ACTIVITY BY ABTS METHOD****Introduction**

Oxidative stress refers to elevated intracellular levels of oxygen radicals that cause damage to lipids, proteins, and DNA. Reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, are one of the highly active oxygen free radicals. During evolution, most living organisms possess enzymatic defenses (superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR)), non-enzymatic antioxidant defenses (glutathione, thioredoxin, Vitamin C, Vitamin E), and repair systems to protect them against oxidative stress. However, these native antioxidant systems are generally not enough to prevent living organisms from oxidative damage. Antioxidant additives using substances that delay or prevent the oxidation of cellular substrates have demonstrated the capacity to protect the human body against oxidative damage. Although several synthetic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been widely used in retarding lipid oxidation, their safety has recently been questioned due to liver damage and carcinogenicity. Therefore, in recent years, finding safer and natural antioxidants from bio-resources to replace synthetic antioxidants has received a great deal of attention. In vitro and in vivo studies indicate that probiotics exhibit antioxidant potential. In this view, consumption of probiotics alone or foods supplemented with probiotics may reduce oxidative damage, free radical scavenging rate, and modification in activity of crucial antioxidative enzymes in human cells. The total antioxidant activity of probiotic candidates can be investigated by means of three potential antioxidant methods; 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical scavenging, ferric reducing ability of plasma (FRAP) assays and 2,2-diphenyl-1-picrylhydrazyl (DPPH).

**Materials****Reagents**

**a) Potassium persulphate solution (140 mM):** 1.892 gm of potassium persulphate was weighed and dissolved in double distilled water and made the volume to 50 ml.

**b) ABTS [2, 2'-azinobis (3 ethyl benzothiazoline)-6-sulfonic acid] stock solution:** 19.2 mg

of ABTS was weighed and dissolved in 5 ml of double distilled water; added 88  $\mu$ l of 140 mM potassium persulphate solution and the mixture was kept in an amber color bottle in dark for 12-16 hours.

**c) Acetate buffer (0.1M, pH 5.0):** Stock solution of acetate buffer was prepared by mixing 148ml of acetic acid solution (0.2M; 11.55 ml of acetic acid in 1000ml distilled water) and 352 ml of sodium acetate trihydrate solution (0.2M; 27.2 gm of sodium acetate trihydrate was dissolved in 1000ml of distilled water) and made the volume to 1000 ml.

**d) ABTS working solution:** 1 ml of ABTS stock solution was diluted with acetate buffer (approx. 1:90) till it gives an absorbance of  $0.70 \pm 0.02$  at 734 nm. The stock solution of ABTS was stable up to 2 days for analytical purpose.

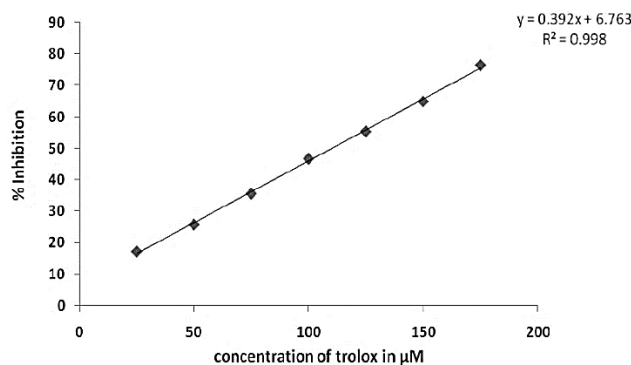
**e) Trolox solution:** 12.5 mg of Trolox [6-hydroxy. 2, 5, 7, 8 – tetramethyl chroman-2-carboxylic acid] was weighed and dissolved in 10 ml of ethanol. The resulting solution was 5 mM trolox solution. It was diluted with distilled water to varying concentrations (25 – 250  $\mu$ M).

#### Preparation of standard curve of Trolox (Vitamin E analogue)

Different volumes from Trolox stock solution (5 mM) were taken in test tubes and the volume of each tube was raised to 2.0 mL with Milli Q water (Table 1). The experiment was conducted in triplicate and repeated twice.

**Table.1. Preparation of Standard Curve of Trolox in ABTS Activity**

Tubes	Blank	1	2	3	4	5	6	7	8	9	10
Distilled Water ( $\mu$ L)	1000	980	960	940	920	900	880	860	840	820	800
Trolox Solution ( $\mu$ L)	0.000	20.0	40.0	60.0	80.0	100	120	140	160	180	200
Trolox Concentration ( $\mu$ M)	0	100	200	300	400	500	600	700	800	900	1000



### **Fig.1. Standard curve of Trolox for ABTS assay**

#### **Procedure**

1. Fermented supernatants from cultures grown in 10% skim milk for 24 hrs. were obtained after centrifugation at 10,000 g/10 min.
2. Working solution of ABTS was prepared by mixing 88  $\mu$ L of 140 mM potassium persulfate solution to 5 mL of 7 mM ABTS stock solution and incubating at room temperature for 16-18 hrs. in amber color bottles for generation of radicals.
3. The absorbance of above generated radical was set to  $0.7 \pm 0.02$  at 750 nm using sterile phosphate buffer saline (approx. 200  $\mu$ L with 40 mL of PBS).
4. Ten  $\mu$ L of standards or samples were pipetted into 96 wells micro-plate and 200  $\mu$ L of working ABTS solution was added to pre-coated wells of micro-plate with help of Finn pipette stepper (12 wells at a time) and the decrease in absorbance was determined each min until 10 min in order to evaluate kinetic behavior of the reaction in Tecan-Infinite F200 PRO plate reader at 750 nm.

#### **Calculation:**

Based on % inhibition of absorbance of sample, Trolox equivalent was determined from standard curve (Fig. 2) using following equation:

$$y = 0.392x + 6.763$$

Where,

y is the % inhibition =  $[(A_{734\text{nm control}} - A_{734\text{nm sample}}) / A_{734\text{nm control}}] \times 100$

x is the  $\mu$ M concentration of Trolox

The results were expressed as Trolox equivalent antioxidant capacity (TEAC) values i.e.,  $\mu$ mol Trolox equivalent /ml of sample extract.

#### **Observations**