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Extraction of ferredoxin from spinach leaves and its effect on *Clostridium perfringens* (anaerobe) growth

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ABSTRACT

In the present work, ferredoxin was extracted from the locally grown spinach plant. The spinach ferredoxin was purified through the Diethylaminoethyl (DEAE) - cellulose column chromatography at 4°C and was sterilized by filtration. Spinach ferredoxin activity was determined by the absorbency measurement (UV –Spectrophotometer) in ultraviolet (UV) - visible region, and with using the total iron content, by the Atomic Absorption Spectrometer. Different concentrations of sterilized spinach ferredoxin were added to the Reinforced Clostridial Agar (RCA) and Cooked meat Media (CMM) as substitutes for cysteine hydrochloride. The amended RCA was then used for cultivation of Clostridium perfringens strain. The growth rate of this bacterium was significantly higher when compared with that on the control medium (RCA anaerobic). The highest growth occurred with 3% spinach ferredoxin added to the RCA. Moreover, the spinach ferredoxin supported the anaerobic bacterial growth even in the lowest concentration (O.O1%). Generally, the tested anaerobic bacterium, Clostridium perfringens produced larger colonies, with regular glistening smooth surface and with irregular or entire edges on media prepared from the spinach ferredoxin, than that on the control medium. This study was targeting to evaluate the importance of spinach-ferredoxin as reducing agent in anaerobic media, liquid and solid, which is more available, cheaper and non-toxic even for human, when compared with cysteine hydrochloride.

Key words: Spinach plant, Ferredoxin, Anaerobic Strain, Growth.

INTRODUCTION

Historically the word ferredoxin was coined to D.C. Wharton of the Du Pont CO. and applied to the iron protein first obtained from *Clostridium pasteurianum*[1]. Compounds from a variety of bacteria and photosynthetic tissues have since been called ferredoxin. All ferredoxin display similar biological activities but show varying degrees of chemical differences [2].

Ferredoxin has the unique property of being the most electronegative carrier yet found in the oxidation-reduction chain in bacteria. Ferredoxin from *C. pasteurianum* has a redox potential (E_0) of –417 mV at pH 7.55 [3]. A related compound has been isolated from spinach leaves and functions in photosynthesis as an electron-trapping agent for the electrons activated by light energy during the photochemical act in the leaf [3] [4].

A common starting material is a water extract of *C. pasteurianum* [1] [3]. Methods of purification and crystallization of ferredoxin have been described by several authors [1] [4] [5]. All workers made use of the specific adsorption of ferredoxin on diethylaminoethyl (DEAE) – cellulose columns for their purification schemes. Ferredoxin is readily separated from most cellular proteins and is isolated as a coffee colored band adhering to the top of a column of DEAE cellulose. Crystalline aggregates which appear as fine brown needles or rosettes.[2].

Spinach Ferredoxin**:**
The ability of bacterial ferredoxin to serve as an electron carrier in the light-induced electron flow of chloroplasts rendered it likely that whole chloroplast contain an endogenous electron carrier with similar properties[3]. For large-scale preparations it was more convenient to purify ferredoxin not from isolated chloroplasts but from an extract homogenate of whole leaves. [6]

Oxidized spinach- ferredoxin is reddish brown in colour. It is reduced (becoming pale) either in the light by chloroplasts or in the dark by hydrogen gas in the presence of hydrogenase. In its reduced form spinach ferredoxin, like *Clostridium* ferredoxin, is very rapidly oxidized by traces of oxygen, and strict anerobicity is therefore required for recording its absorption spectrum.[3] The absorption spectrum of reduced spinach ferredoxin has a marked peak in the ultra-violet region at 267mµ, a shoulder at 312mµ and a broad shoulder between 450 and 470 mµ. In oxidized form absorption peaks were observed at 463, 420, 325 and 274 mµ [3] [2].

The absorption spectrum of spinach ferredoxin showed both similarities with and differences from the absorption spectrum of *Clostridium* ferredoxin. The most marked similarities are the pronounced absorption peaks around 260 mµ in the reduced form and a difference peak around 415 mµ.

The redox potential of spinach ferredoxin was found to be some what more electronegative ((E_0) of –432mV at pH 7.55) than that of *Clostridium* ferredoxin. Spinach ferredoxin is the most reducing constituent isolated thus far from the photosynthetic apparatus of green plants or photosynthetic bacteria [3].

The preliminary analyses of spinach ferredoxin showed relatively high iron content (0.815 percent) and a ratio of ferredoxin to chlorophyll of about 1:400. The same ratio was obtained whether the determination was made on whole leaf material or isolated whole chloroplasts, showing that ferredoxin are localized in chloroplasts. An elemental analysis gave, in percent, C, 43.47; N, 14.15; H_2, 6.32 and ash residue of 3.5, [3] [7]

MATERIALS AND METHODS

1. Spinach ferredoxin:
i) Samples preparation:
Spinach leaves purchased from the local market of Khartoum state (Sudan), winter season, were washed thoroughly with tap water, torn by hand into small shreds (the mid rib and large veins were discarded). Then they were used freshly or stored frozen in plastic bags for one year.

ii) Preparation of the buffer:
The buffer solutions used in this study were prepared according to [8] method. It consisted of:
i. Potassium phosphate buffer 0.15 M, pH 7.3.
ii. Potassium phosphate buffer, 0.05 M, pH 6.5.
iii. Tris (hydroxymethyl) aminomethane, HCl, buffer pH 7.3, 0.15 M.
iv. Tis HCl buffer, pH 7.3, 0.7 M.
v. Phosphate buffer (KH_2PO_4 1.0 M + NaOH, 1.0 M, pH 6.5).
vi. Phosphate buffer (KH_2PO_4 0.2 M+ NaOH, 0.2 M, pH 6.5).
vii. Phosphate buffer (KH_2PO_4 1.0 M+ NaOH, 0.2 M, pH 6.5)

iii) Determination of spinach leaves solvent:
A total of 19 trials were carried out to determine the suitable solvent for isolation of ferredoxin from spinach leaves. These were as follows:
1. 50 g of fresh spinach whole leaves + 100 ml ice-cold water.
2. 100 g of fresh spinach whole leaves + 250 ml ice-cold water.
3. 150 g of fresh spinach whole leaves + 350 ml ice-cold water.
4. 25 g of fresh spinach leaves + midribs + 200 ml water.
5. 25 g of spinach roots and midribs + 250 ml water.
6. 25 g of fresh spinach leaves only + 200 ml ice-cold potassium phosphate buffers 1.0 M, pH 6.5
7. 25 g of a frozen spinach leaves + potassium phosphate buffer.

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8. 25 g of fresh rocket leaves + 200 ml ice-cold water.
9. 80% Acetone extract after purification.
10. 25 g of frozen spinach + phosphate buffer (KH_2PO_4 1.0 M + NaOH, 1.0 M, pH 6.5).
11. 230 g frozen spinach + phosphate buffer 500 ml (KH_2PO_4 Lom + NaOH, 1.0 M, pH 6.5).
12. 12.5 g frozen spinach leaves + 100 ml ice-cold water extracted and centrifuged in 2/2/99 and examined after 6 months.
13. 100 g fresh spinach leaves + 250 ml ice-cold water, firstly extracted on 2/2/1999 and kept in a freezer till 19/7/1999 where it was reextracted, centrifuged and examined.
14. 12.5 g frozen spinach leaves + 100 ml ice-cold water.
15. 12.5 g frozen spinach leaves + 100 ml buffer (K_2HPO_4 + KH_2PO_4, 1.0 M, pH 6.5).
16. 12.5 g frozen spinach leaves + 100 ml buffer (KH_2PO_4 0.2 M + NaOH 0.2 M, pH 6.5).
17. 12.5 g frozen spinach leaves + 100 ml buffer (KH_2PO_4 1.0 M + NaOH 0.2 M, pH 6.5).
18. 12.5 g frozen spinach leaves + 100 ml tris buffer 0.15 M, pH 7.3.
19. 50 g frozen spinach leaves + 100 ml water, firstly extracted on 2/2/1999 and kept in a freezer till 27/7/1999 where it was centrifuged.

The above samples from 1 to 19 were blended using a Waring blender, filtered through a cotton cloth, centrifuged at 18,000 xg. for 20 min. at 4°C.The supernatant from each extract was collected and the precipitate was washed with 100 ml of water by centrifugation and discarded. The wash water was added to the initial extract.

iv) Spinach ferredoxin isolation and purification:
The method used for isolation and purification of ferredoxin form spinach leaves, was essentially the same method which was found effective for *Clostridium* ferredoxin, [1] [9] .All of the purification procedures were done at 3-4°C.

v) Determination of ferredoxin activity:
Spinach ferredoxin activity was determined for the first 9 samples by absorbency measurement, using the Ultraviolet (UV) spectrophotometry [2], while for the samples 10 to 19, the activity was determined using the total iron content, using the Atomic Absorption Spectrometry [10] [4].

2. Gel chromatography purification of spinach ferredoxin:
i) Preparation of spinach extract:
240 grams of spinach leaves (fresh or frozen), were washed, torn (midribs were discarded) and ground with 250 ml of ice-cold acetone 80% solution, [11]. Grinding was carried out in a Warring blender for 3 minutes. The homogenate was filtered through a thick cotton fabric (Domoria) to remove the coarse materials, and the filtrate was centrifuged for 20 min at 4°C at a speed corresponding to approximately 18,000,xg in a International Refrigerated Centrifuge. The supernatant was decanted, the precipitate from the high speed centrifugation was washed with 100 ml ice-cold distilled water, centrifuged again and discarded. The wash water was added to the initial extract.

The final extract was freed from salts by dialysis against distilled water for 48 hrs at 4°C using a magnetic stirrer. The dialyzed extract was supplemented with 1/100 volume of 0.7 N tris hydrochloric acid buffer, pH 7.3, and solid sodium chloride 0.2N. Five ml was taken for activity determination and the rest was kept in the refrigerator for further purification.

ii) Gel filtration:
Gel filtration on DEAE-cellulose was used for purification of spinach ferredoxin, following the procedure adopted by[1]. All steps of the purification were done at 3-4°C.The gel was prepared according to the methods described by [1] [11]

iii) Sample application to the column (Pharmacia column R25/26) [11]
The outlet tubing was closed and the buffer above the gel surface was removed by sucking. The outlet was then opened and the remaining buffer was allowed to drain away. Spinach extract was applied on the top of the equilibrated column using STA-Pris taltic pump. A sample applicator cup was routinely used to prevent disturbance of the bed surface. The column outlet was then opened to allow the sample drain into the bed, at a constant flow rate of 50 ml/cm^2/hr. Then the flow rate was reduced by about one half of the packing rate during elution. The major portion of the protein, about 95% representing phosphoroclastic system, minus ferredoxin, passed directly through the column. A dark bond containing ferredoxin remained absorbed at the top of the column which was washed with ten volumes of distilled water until the elute was colorless followed by 10 volumes of 0.05M potassium phosphate buffer, pH 6.5, to remove unwanted proteins. The ferredoxin was then eluted with 0.15M tris HCl buffer, pH 7.3, containing 0.67 M sodium chloride, in a volume of 120 ml [4].

iv) Concentration and sterilization:
200 ml aliquots of the elute containing ferredoxin obtained in the previous step, was concentrated to 50% by dialysis against polyethylene powder, overnight, at 4°C. The concentrated ferredoxin was sterilized by filtration using a membrane filter (0.2 µm) (Gelman Sciences). The filtrate was collected aseptically in sterile bottle, and stored at 4°C for use [12]

3. Effect of spinach ferredoxin on the growth of anaerobic bacterium:
Strain
Strain used in this study was:
Air tolerance *Clostridium perfringens*, obtained from the microbiology lab.in the Faculty of Veterinary Medicine, University of Khartoum, Sudan, Table (1)

Table (1) Bacterial strain used in this study

No	Bacteria	Family	Main Medical Importance
1	*Clostridium perfringens*	Bacillaceae	There are six types of *C. perfringens* (A, B, C, D, E, F). Human disease is caused by type A, type C, and occasionally by typep[13] F. *C. perfringens* type A causes gas gangrene (myonecrosis), food poisoning, pnerperal infection and septicemia[14].

i) Morphology and purification:
Clostridium. perfringens strain was grown on Reinforced Clostridial Agar (RCA, Oxoid) and blood agar media for purification and study of colonial morphology. Smears were made from culture media and stained with Gram's stain. Reinforce Clostridial Agar (RCA) was prepared with the same procedure described in [12]

ii) Effect of spinach-ferredoxin on the growth of *C. perfringens:*
Reinforced Clostridial Agar was prepared with different concentrations of spinach- ferredoxin and cysteine hydrochloride as follows:
(Cysteine 0.00%, 0.005%, 0.05% and ferredoxin, 0.00% 1%, 2%, 3%, 4% and 5%).

A 5% stock solution of cysteine hydrochloride (Analar) was prepared and sterilized by filtration using 0.45µ Seitz filter. Cysteine was then incorporated in RCA at different concentrations using the formula[12] :

Volume of cysteine required per 100 mls of RCA =

$$\frac{\text{Concentration of cysteine wanted}}{\text{Concentration cysteine in stock solution}} \times 100$$

iii) Preparation of modified RCA:
Different concentrations of spinach ferredoxin and cysteine hydrochloride, were added to sterile RCA media aseptically, and poured on to sterilized petri dishes and stored at 4°C for use. These concentrations were as follows:
1. RCA without cysteine + 1% ferredoxin.
2. RCA without cysteine + 2% ferredoxin.
3. RCA without cysteine + 3% ferredoxin.
4. RCA without cysteine + 4% ferredoxin.
5. RCA without cysteine + 5% ferredoxin.
6. RCA without cysteine + 0% ferredoxin.
7. RCA without cysteine + 0.01% ferredoxin.
8. RCA + 0.005% cysteine + 1.0% ferredoxin.
9. RCA + 0.05% cysteine.
10. RCA + 0.005% cysteine.
11. RCA optimum medium (control).

iv) Inoculation:
The modified RCA in (3.7.2.4.1) and the RCA (control) media were inoculated using a loopful of a pure overnight culture of *Clostridium perfringens* using the Streak Plate Procedure. The same modified RCA and the optimum RCA (control) plates were inoculated by one drop (0.02 ml) of *C. perfringens*, incubated overnight in cooked meat media, spread by a sterile glass rod. Duplicate plates were incubated anaerobically at 37°C for 24 hrs, using Baird and Taltock anaerobic jar (BTL) containing a room temperature catalyst (Deoxy pellets, Engel hard industries, Gauderford, GIOS) and the Gaspak system (BBL, Oxoid). Smears were made after the jars were opened. These were fixed and stained with Gram's stain, and examined by light microscopy. Morphology of cells was studied and selected fields were photographed, and colonial morphology were studied and photographed.

v) Crystalline spinach ferredoxin:
One drop of sterile spinach ferredoxin was spread on a clean glass slides fixed by heat, and examined under light microscope, 40 powers. Selected fields were photographed[5].

RESULTS

Determination of spinach-ferredoxin (spin-fd) solvent and its activity:
The results of solvent determination for spinach-ferredoxin showed that the highest iron content was observed in spinach leaves extracted with water (Fig 1).

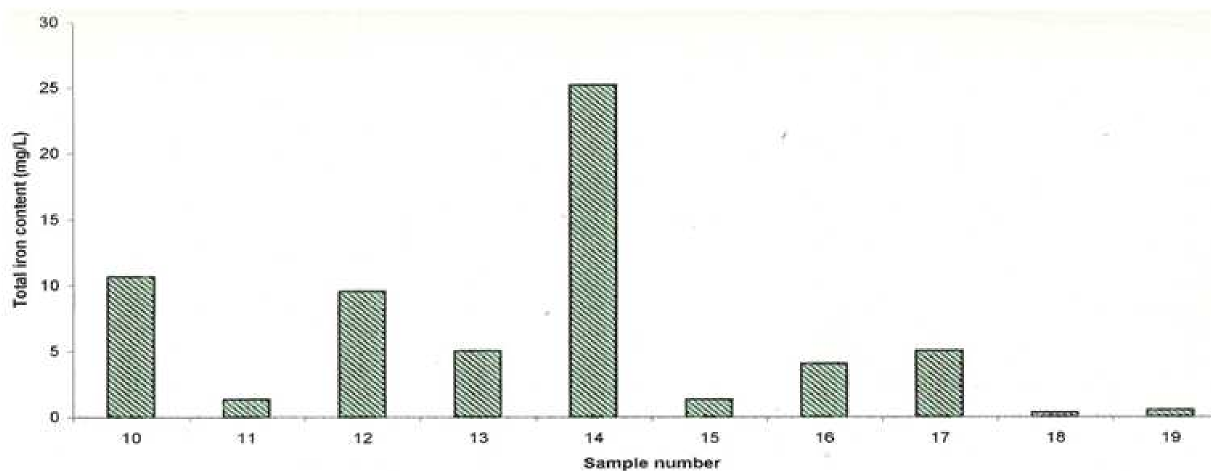


Fig. (1): Solvent selection for spinach ferredoxin and its activity

Gel chromatography:
Spinach-ferredoxin purification, through the diethylaminoethyl (DEAE) cellulose column, was done at 3°C - 4°C as shown in plate (1).

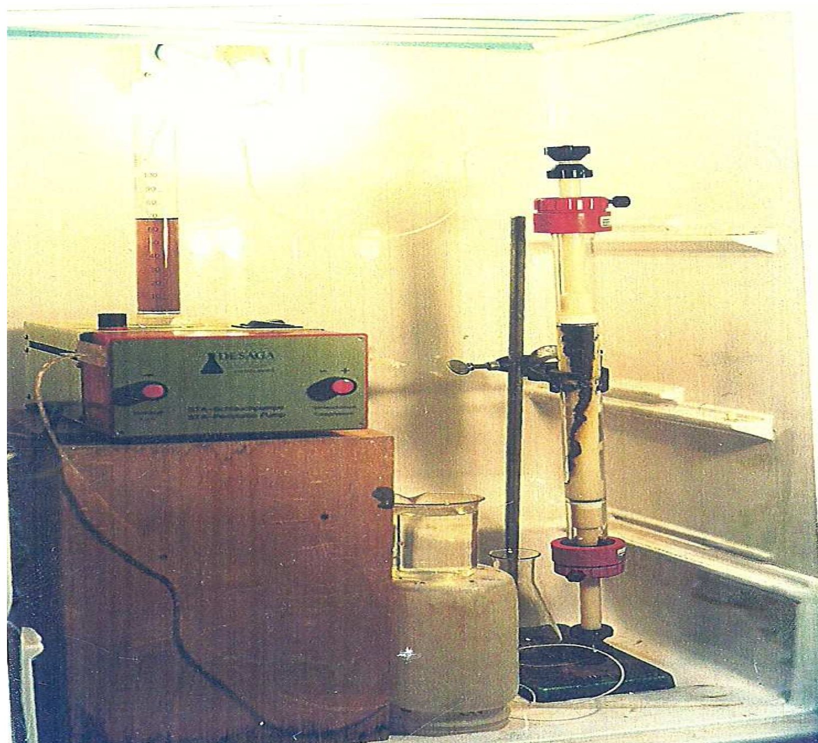


Plate (1): Spinach- ferrdoxin band on DEAE cellulose column

Spectral properties of spinach-ferredoxin (spin-fd)

As shown in Fig 2, Fig 3, and Fig 4, the absorption spectra of crude spinach-ferredoxin extracted from the whole leaves in ice-cold water had marked peaks in the ultra-violet regions 268, 276 and 274 mμ and shoulders at 312, 314 and 312mμ respectively.

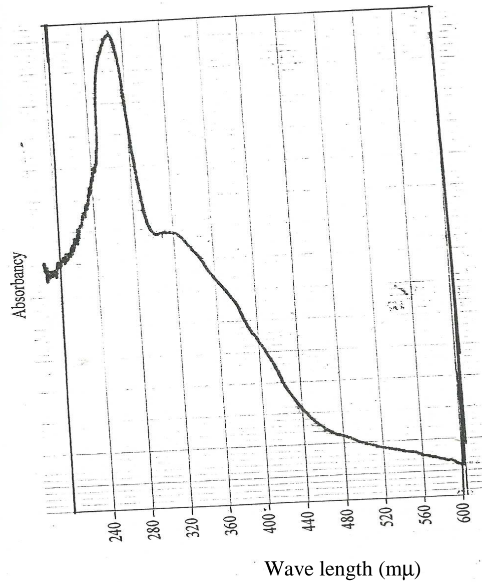


Fig. (2): Absorption spectrum of spinach-ferredoxin sample (1)

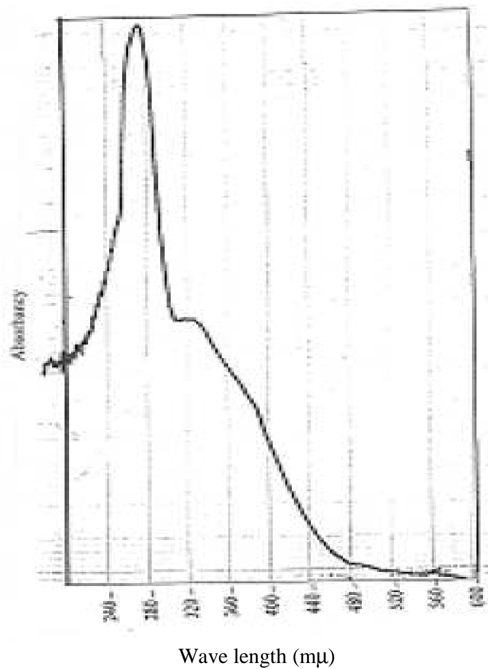


Fig. (3): Absorption spectrum of spinach-ferredoxin sample (2)

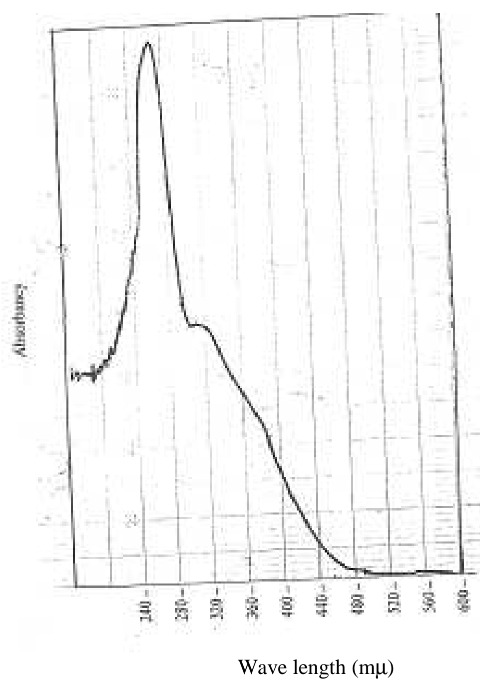


Fig. (4): Absorption spectrum of spinach-ferredoxin sample (3)

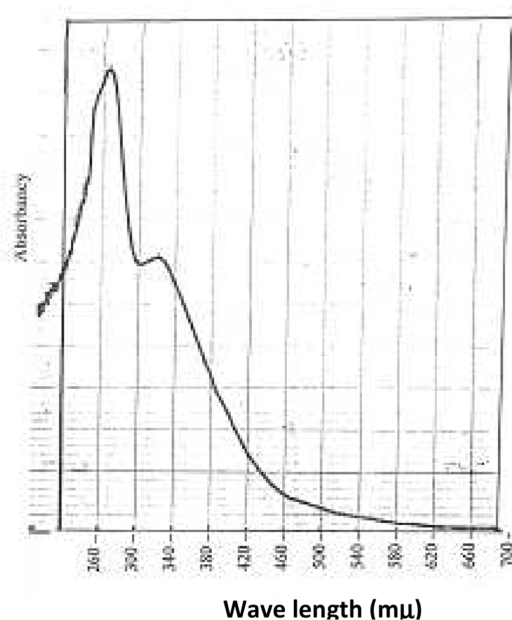


Fig. (5): Absorption spectrum of spinach-ferredoxin sample (4)

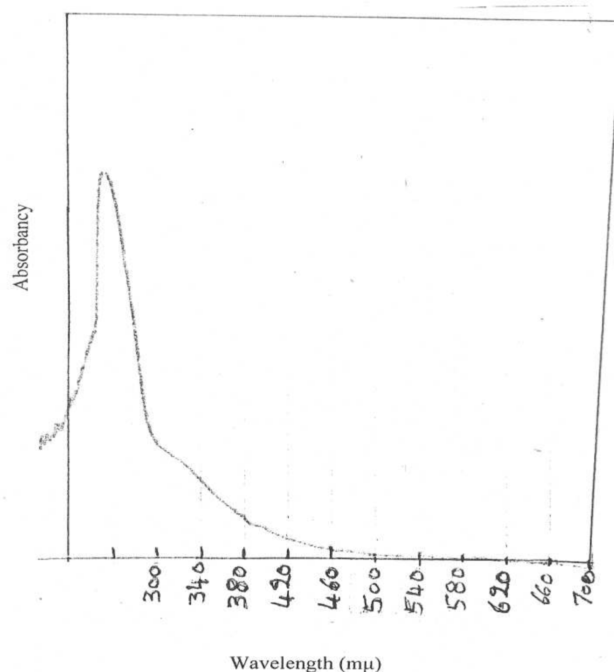


Fig. (6): Absorption spectrum of spinach-ferredoxin sample (5)

The absorption spectrum of crude spinach-ferredoxin extracted from the whole leaves + midribs, in ice- cold water (Fig 5) had a peak in the ultra-violet region at 268 mμ and a shoulder at 320 mμ.In contrast the spectrum of spinach extracted from roots + midribs in ice- cold water which consisted of a single peak at 260 mμ indicating that no ferredoxin content (Fig. 6).here

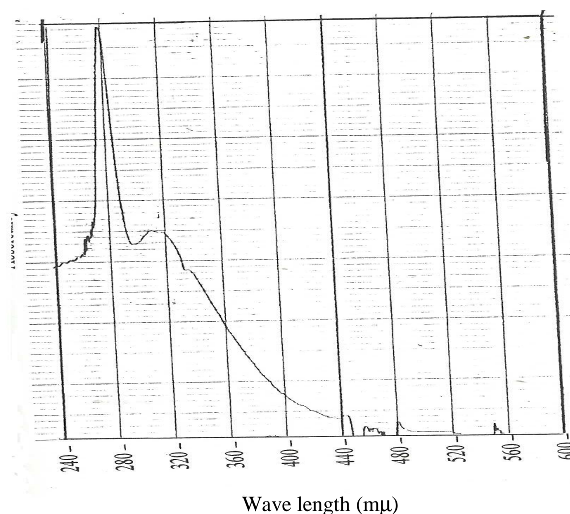


Fig. (7): Absorption spectrum of spinach-ferredoxin sample (6)

Fig 7 and Fig 8 showed the absorption spectra of the crude spin-fd extracted from frozen leaves in ice- cold potassium phosphate buffer. They showed marked peaks in the ultra-violet regions at 268, 292 mμ and shoulders at 325 and 344 mμ respectively.

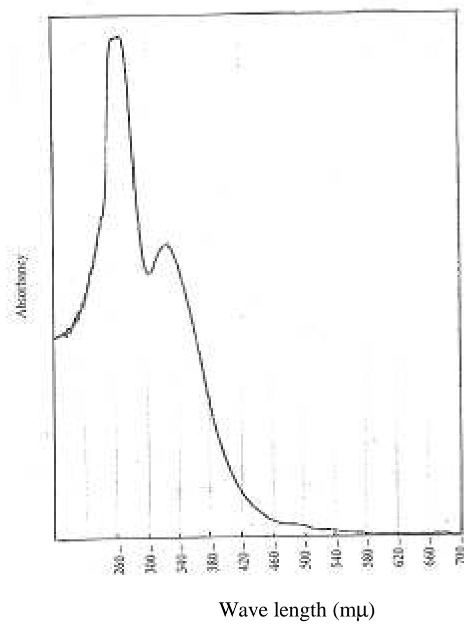


Fig. (8): Absorption spectrum of spinach-ferredoxin sample (7)

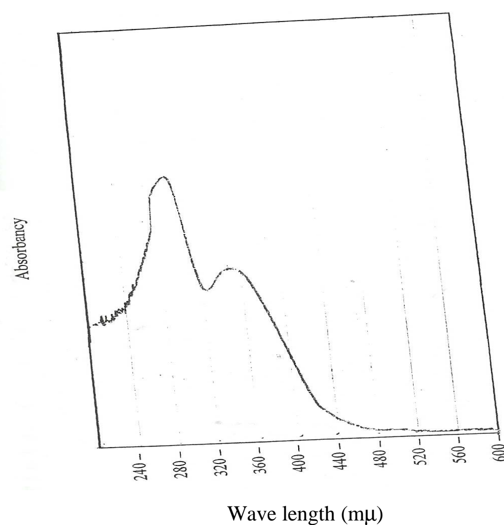
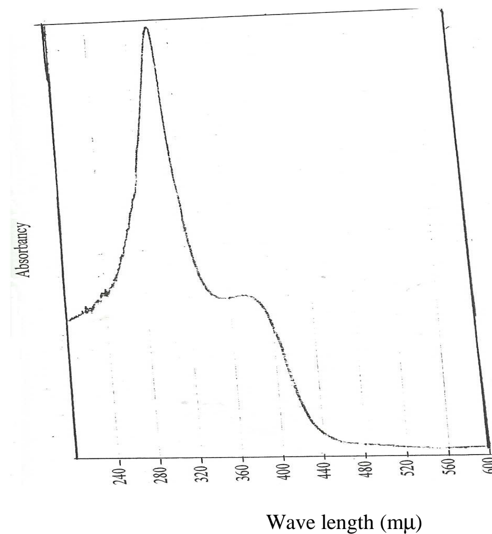


Fig. (9): Absorption spectrum of spinach-ferredoxin sample (8)

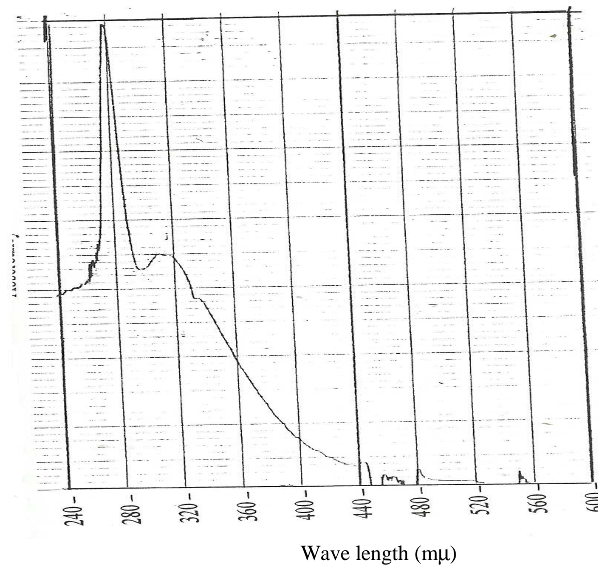
The absorption spectrum of crude garden-rocket extracted with water had a peak in the ultra-violet region at 296mμ and a shoulder at 372mμ (Fig 8).



Wave length (mµ)

Fig. (10): Absorption spectrum of spinach-ferredoxin sample (9)

However the absorption spectrum of the purified spin-fd extracted from whole leaves in 80% ice-cold acetone water had a marked peak in the ultra-violet region at 274mµ and a shoulder at 312mµ (Fig10).



Wave length (mµ)

Fig. (11): Absorption spectrum of spinach-ferredoxin sample(10)

Growth of C. *perfringens* on amended Reinforced Clostridial Agar media (RCA) and RCA (control):
There were variations in the growth of *C. perfringens* on the amended RCA media. The growth on media 1(no cyst + 1% spin-fd) and 2 (no cyst + 2% spin-fd) was profuse where on medium 3 (no cyst + 3% spin-fd) the growth was condense, more profuse and the colonies were larger than on RCA the optimal medium (control) (plates 2,3 and 4)

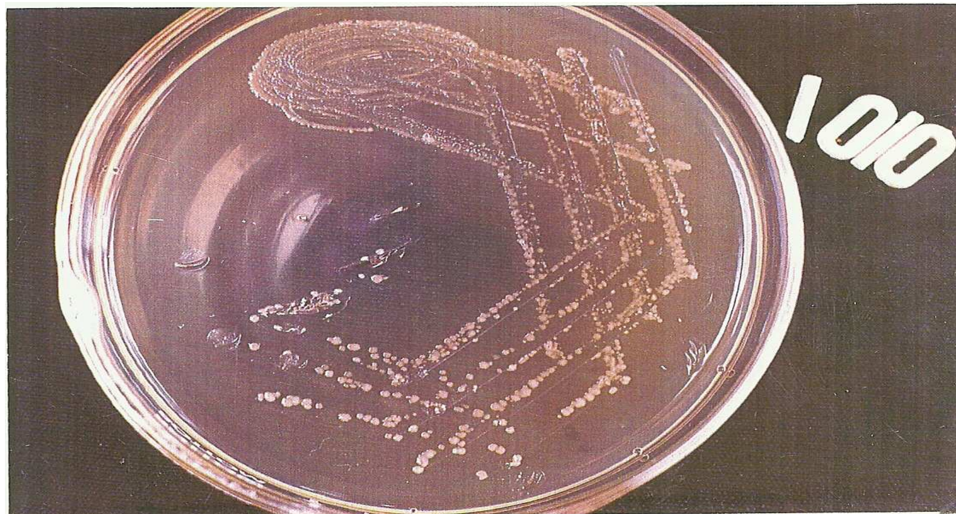


Plate (2): Growth of *Clostridium perfringens* on amended RCA (no cys 1% spin-fd) after 24 hrs incubation at 37°C)

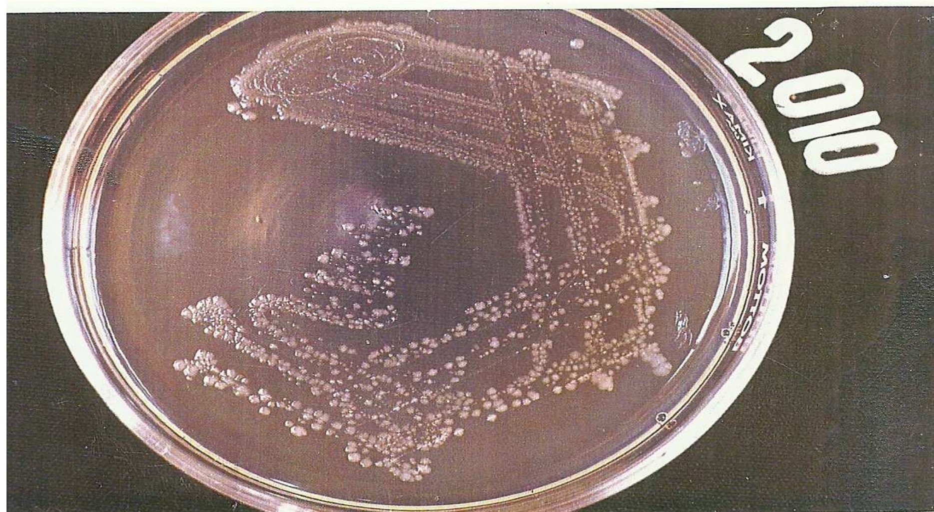


Plate (3): Growth of *Clostridium perfringens* on amended RCA (no cys 2% spin-fd) after 24 hrs incubation at 37°C)

The growth on medium 4 (no cyst + 4% spin-fd) and 5 (no cyst + 5% spin-fd) was less than that on the previous media (plates 5and 6).

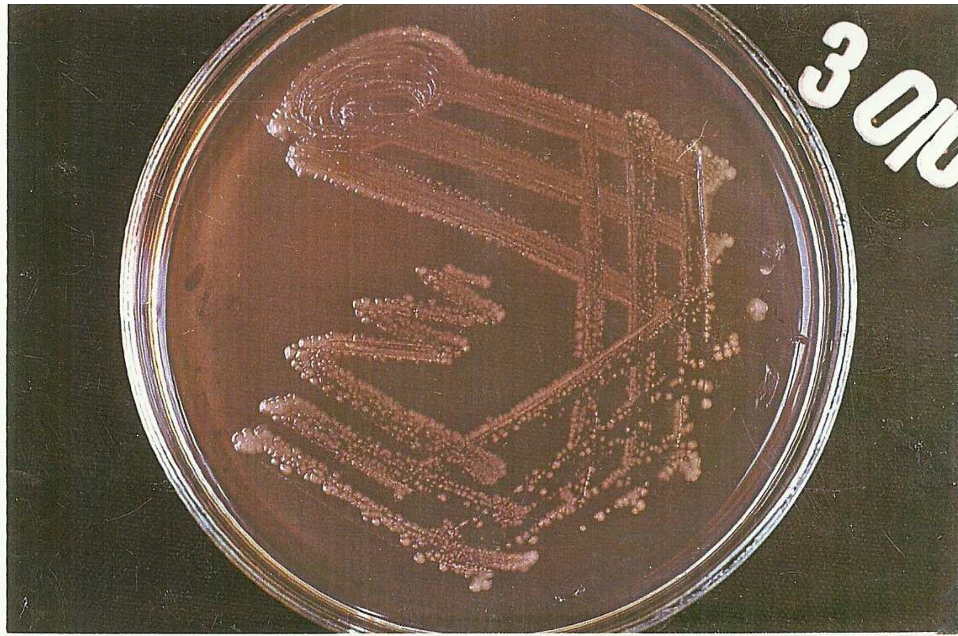


Plate (4): Growth of *Clostridium perfringens* on amended RCA (no cys 3% spin-fd) after 24 hrs incubation at 37°C)

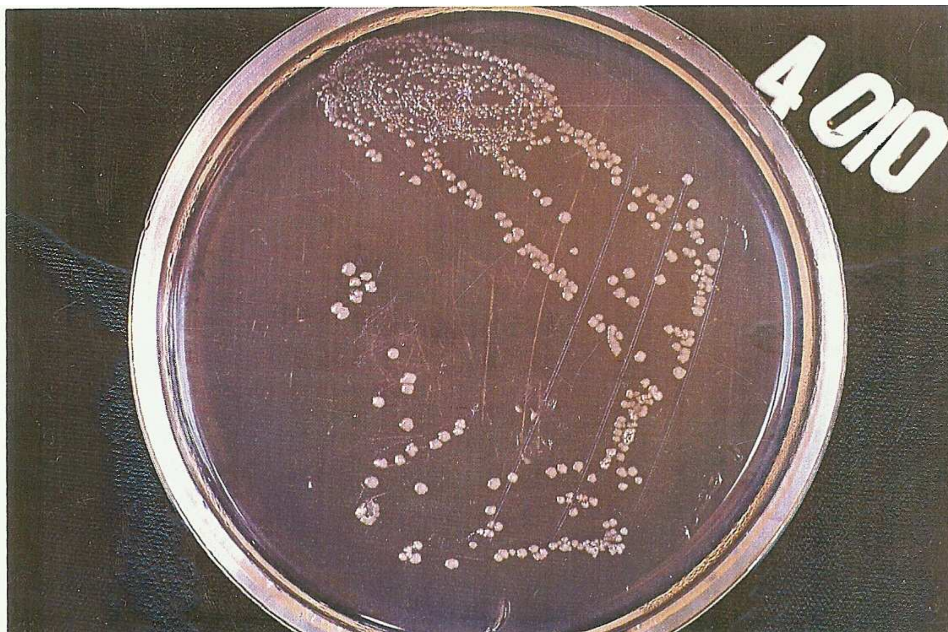


Plate (5): Growth of *Clostridium perfringens* on amended RCA (no cys 4% spin-fd) after 24 hrs incubation at 37°C)

C. perfringens growth on medium 6 (no cyst and no spin-fd) was poor and sparse, but on medium 7 (no cyst + 0.01% spin-fd) it was better than on RAC (11) medium (control).

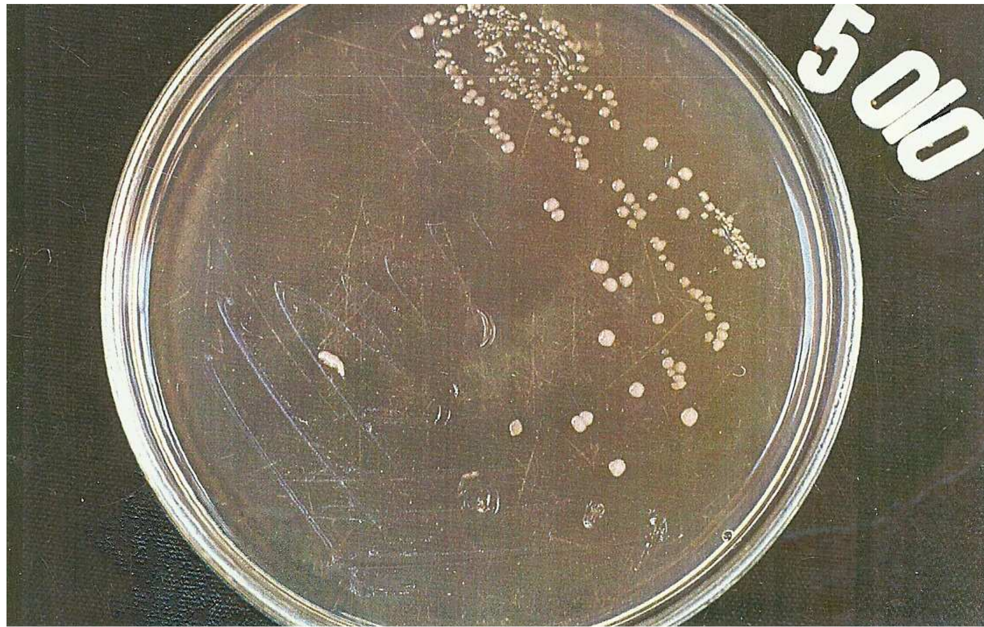


Plate (6): Growth of *Clostridium perfringens* on amended RCA (no cys 5% spin-fd) after 24 hrs incubation at 37°C)

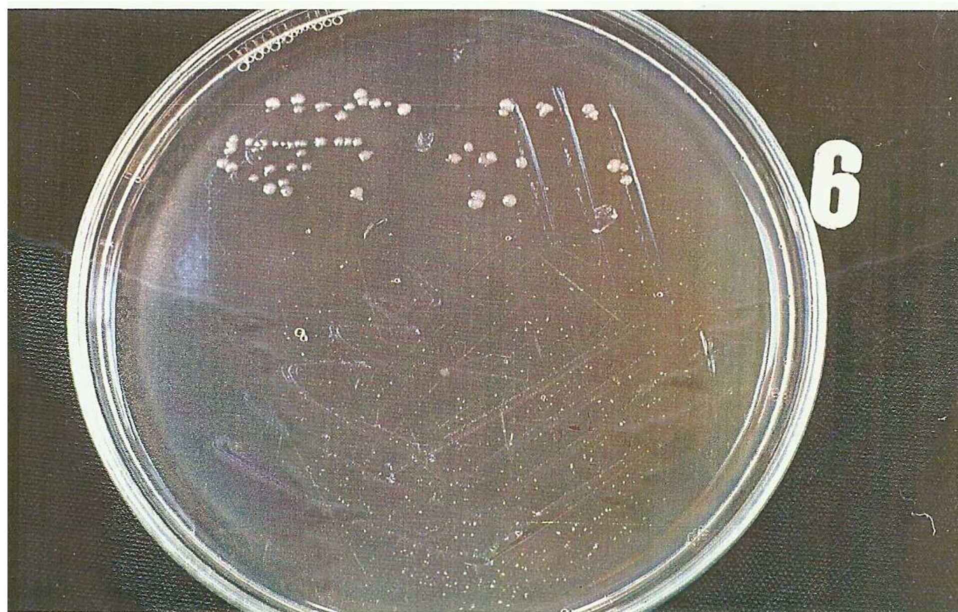


Plate (7): Growth of *Clostridium perfringens* on amended RCA (no cys, no spin-fd) after 24 hrs incubation at 37°C)

On media 8 (0.005 cyst + 1.0% spin-fd), 9 (0.05 cyst) and 10 (0.005 cyst) the growth was semi-dense and the colonies were larger. The growth on RAC (11) optimum medium (control) was poor and the colonies was minute (plates7,8,9,10,11and 12 control).

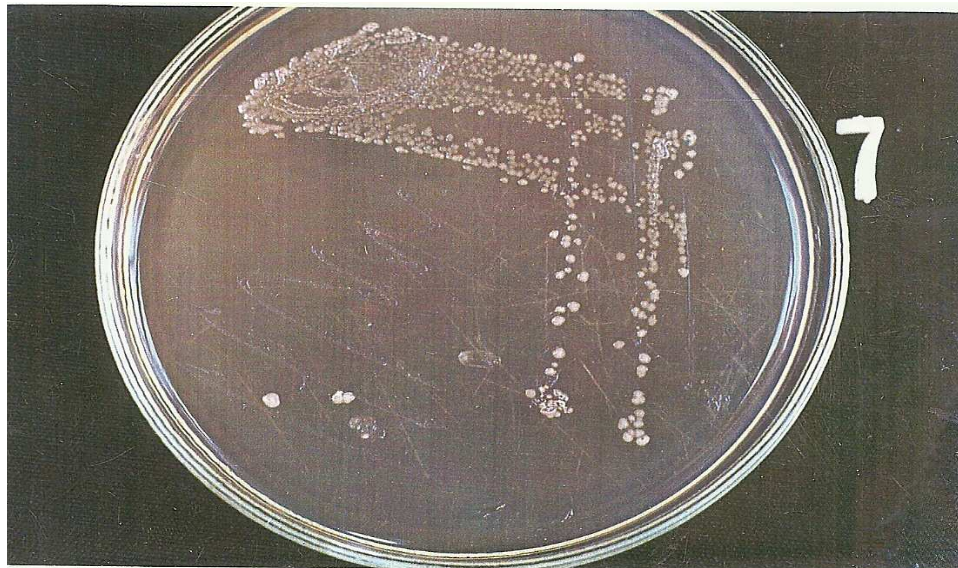


Plate (8): Growth of *Clostridium perfringens* on amended RCA (no cys, + 0.01% spin-fd) after 24 hrs incubation at 37°C)

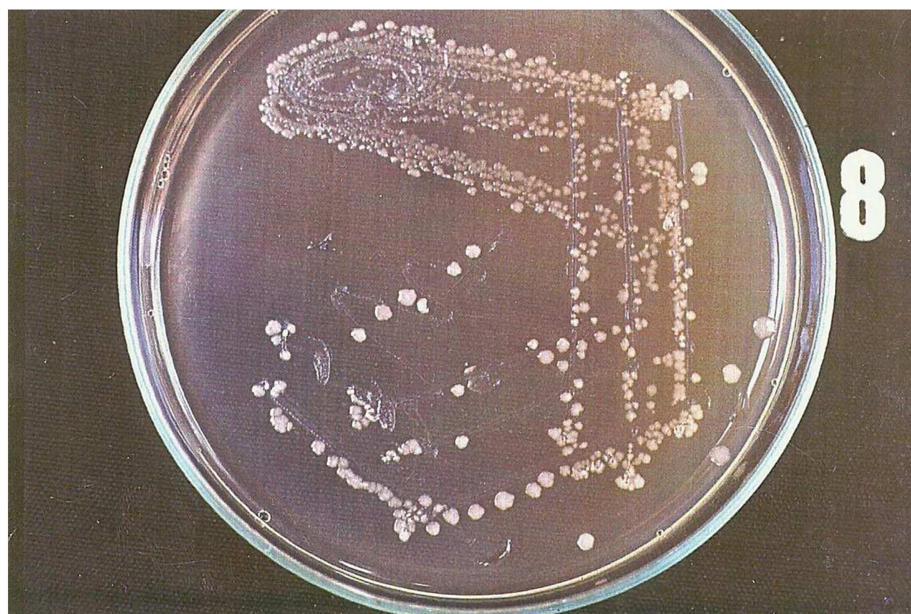


Plate (9): Growth of *Clostridium perfringens* on amended RCA (0.005% cys, + 1.0% spin-fd) after 24 hrs incubation at 37°C)

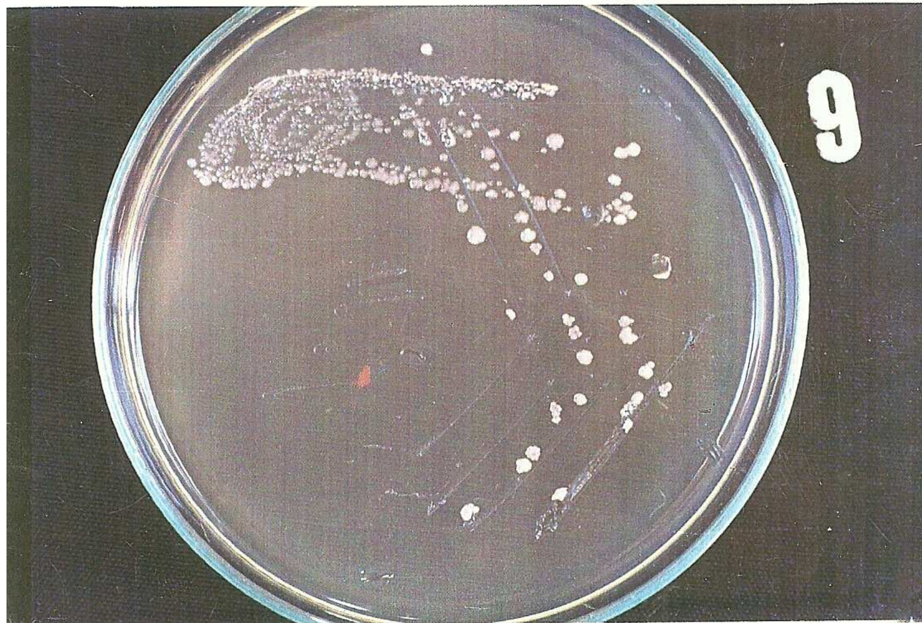


Plate (10): Growth of *Clostridium perfringens* on amended RCA (0.05% cys. no spin-fd) after 24 hrs incubation at 37°C)

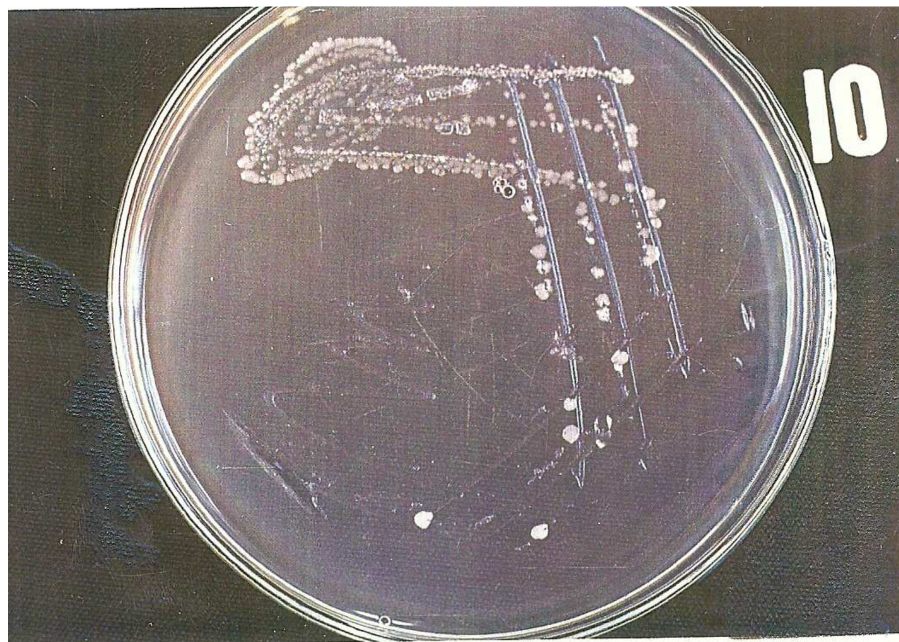


Plate (11): Growth of *Clostridium perfringens* on amended RCA (0.005% cys, no spin-fd) after 24 hrs incubation at 37° C

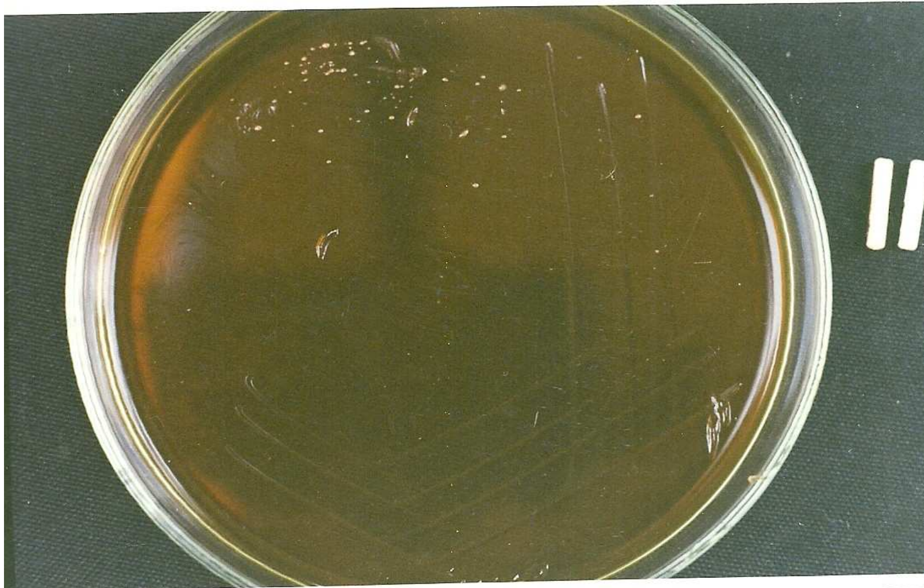


Plate (12): Growth of *Clostridium perfringens* on amended RCA (control) after 24 hrs incubation at 37°C

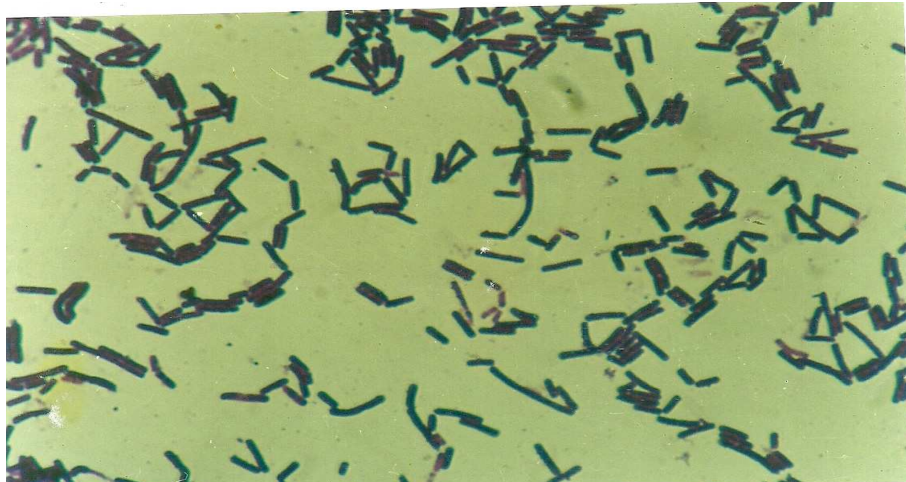


Plate (13): *Clostridium perfringens* in Gram-stained smear from growth on fresh RCA (no cys. + 1% spin-fd) culture (x 100)

i. Colonial morphology: *Clostridium perfringens* produced circular colonies with different sizes, about 2-4mm large and with regular glistening smooth surface with irregular or entire edges (Plates 2…12).

ii. Cell morphology: Microscopic examination of *C. perfringens* revealed more or less long and thick Gram-positive rods, with rounded ends, which were encapsulated. Rods arranged singly and in pairs. Short chains were seen. It is clear that *C. perfringens* rods produced on the amended RAC media were longer and thicker than those on RAC (11) control medium (plates13, 14 and15) and (plate16 control)

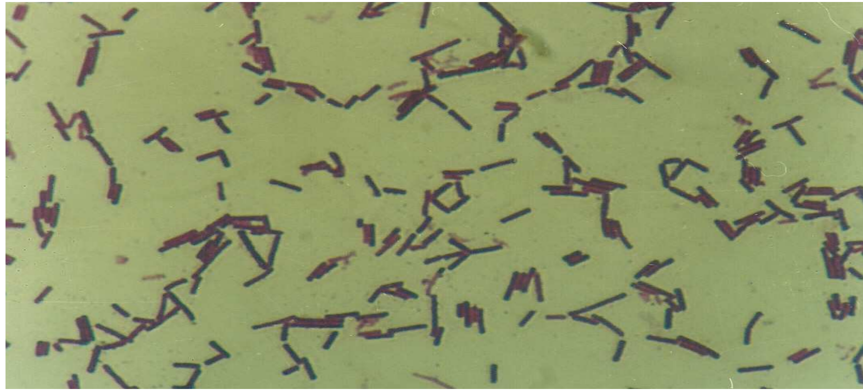


Plate (14): *Clostridium perfringens* in Gram-stained smear from growth on fresh RCA (no cys. + 2% spin-fd) culture (x 100)

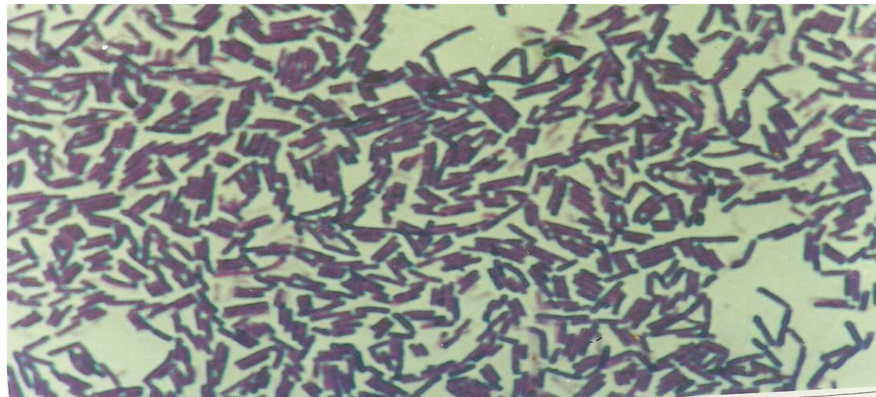


Plate (15): *Clostridium perfringens* in Gram-stained smear from growth on fresh RCA (no cys. + 3% spin-fd) culture (x 100)

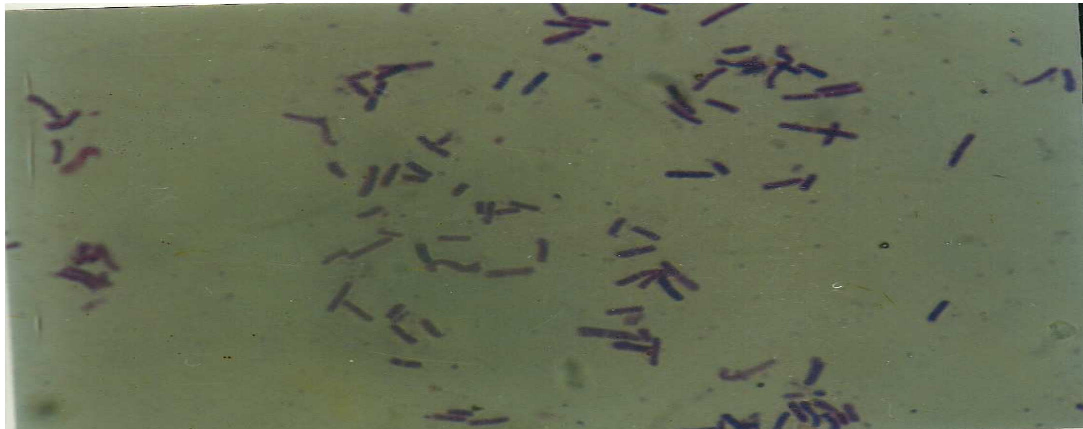


Plate (16): *Clostridium perfringens* in Gram-stained smear from growth on fresh RCA (control) culture (x100)

Spinach-ferredoxin crystallization:
Photomicrograph of spinach-ferredoxin was shown in plate (17). The crystals were brown in colour and formed needles shape.

Plate (17): Photomicrograph of Spinach-ferredoxin crystals

DISCUSSION

Spinach-ferredoxin:

In this investigation the results of the spinach-ferredoxin (spin-fd) solvent determination, indicated that water extract released relatively high iron content. These findings substantiated the work of [3] and [4[. As shown in Figs (2, 3,4 and 10), the absorption spectra of spinach-ferredoxin obtained in this study are similar to the results reported by [3]. The absorption spectra of purified and crude spinach-ferredoxin showed similarities with the findings reported by [3]. The most marked similarities are the pronounced absorption peaks around 260 to 274 mμ and shoulders around 312 to 325 mμ in the oxidized form (exception was noticed in rocket absorption spectrum Fig 9). The results of this study showed the similar findings reported by [3] that the spinach-ferredoxin was found in the whole leaves. However, another finding was recorded in this investigation indicating that spinach roots and midribs containing no ferredoxin.

Effect of spinach-ferredoxin on the growth of *Clostridium perfringens* has been investigated. In the previous literature [13] found that ferredoxin freed from *C. pasteurianum*, serves as source of electrons for conversion of molecular nitrogen to ammonia. Also may function as an electron carrier for several ferredoxins - dependant oxidation – reduction reactions in clostridia [2].

In this study the effect of addition different concentrations of spin-fd to Reinforced Clostridial Agar media (RCA), for the growth of *C. perfringens* was investigated. The results indicated that the amended (RCA) media which supplemented with 1% and 2% spin-fd, showed better and dense growth. The most significant result was obtained by the addition of 3% spin-fd to the amended (RCA) plate (4). The *C. perfringens* growth was profuse and very condenses and the colonies were larger than on the RCA control. This might be due to the fact that spin-fd is an active electron-carrier, non toxic and supported the bacterial growth. The media with 4% and 5% spin-fd added, the growth rate was relatively less than on the previous media and greater than on RCA control. This may be explained by the fact that increasing of spin-fd concentration, more than 3% could have a toxic effect.

Medium (6) which was free of spin- fd and cysteine hydrochloride produced a poor and weak growth for *C. perfringens* (*C. perfringens* is a relatively oxygen tolerant), because the medium is lacking the reducing agent. Even in the lowest spin-fd concentration i.e. 0.1% and no cysteine hydrochloride added to RCA medium (7), but *C. perfringens* showed better growth. This indicated the significance of spin-fd added. Another finding was obviously noticed in medium (8) which contained both cysteine hydrochloride 0.005% and spin-fd 1%, produced significantly better growth compared to its growth on RCA the optimum medium. This may be due to the fact that, there is a synergistic action between the two chemical for the support of *C. perfringens* growth.

The *C. perfringens* growth on media (9) and (10) which contained 0.05% and 0.005% cysteine hydrochloride, respectively was better but was dense on (9), with 0.05% cysteine hydrochloride which is the optimum concentration in the RCA. The growth decreases on medium (10) with 0.005% cysteine hydrochloride. Because it is less than the *C. perfringens* requirements to grow. *C. perfringens* colonies on all the amended RCA were larger than those on the RCA optimum medium. However, the growth on the RCA control (11) is weak, poor and sparse. The colonies were very minute plate (12).

The microscopic examination for *C. perfringens* cells showed obvious differences between cell morphology in the amended RCA and RCA control. In the amended media the cells were large Gram-positive with rounded ends plates (13, 14 ,15and16). But on the RCA control the cells were shorter with rounded ends plate (17). These differences in the cells morphology may be due to the different concentration of spin-fd and cysteine hydrochloride added. These findings were in agreement with the observations of [14] and [15] who reported that the different concentrations of cysteine hydrochloride affect the bacterial growth.

Crystallization of Spinach-Ferredoxin:
Spinach-ferredoxin crystals are of brown colour and needles shape. This result is matching with that of [5] who reported the similar findings from the crystallization of ferredoxin released from *C. acidi-urici*.

CONCLUSION

1. In this investigation Spinach-ferredoxin supplementation to the RCA in the place of cysteine hydrochloride, showed obvious increase in *C. perfringens* growth the maximum growth was obtained at 3% of spin-fd added. Even small amount of spin-fd supported anaerobic bacterial growth.
2. Spinach-ferredoxin was located in the whole leaves and not found in the roots and midribs of spinach plant

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