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PROCESS AND DEVELOPMENT FOR THE PRODUCTION OF BIOACTIVE METABOLITES FROM *Cordyceps militaris*

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Abstract

Cordyceps militaris is a really high valued medicinal fungus and has been used since historical instances for application for revitalization of numerous structures of the human body. Due to the fact C. militaris can be grown in any lifestyle and has good medicinal impacts, marketplace call for for the synthesis of synthetic C. militaris has accelerated a selection of culture techniques mentioned especially for this mushroom which includes storage stock culture, pre tradition, laboratory subculture such as shaking culture, submerged culture , surface liquid culture and continuous batch subculture, cultures with diverse substrates like brown rice, rice, wheat, sprouts, corn and so on. The most fulfilling substrate for harvesting the species is brown rice. The water-soluble crude polysaccharides were acquired from the fruiting bodies of cultured Cordyceps militaris via hot water extraction, Cordycepin is aantibiotic extracted from the fungus Cordyceps militaris having various antineoplastic, antioxidant, and anti-inflammatory activities .

Effect of ph, unique substrate, time direction, substrate concentration and light on cordycepin production was studied at different culture conditions. Regular design was used to optimize the extraction of cordycepin from cultured *Cordyceps militaris*. The concentration of cordycepin identified using high performance liquid chromatography (HPLC) at a detection wavelength of 210 nm.

Keywords: fungus, cordycepin, stock cultures, Hplc

1. Introduction

The fruit body of the Cordyceps militaris mushroom originates at its base on an insect larval host (typically the larva of the Himalayan bat moth, , although once in a while other insect hosts except the bat moth are encountered and ends at the club-like cap, 6 includes the stripe like stomata. The fruiting body is dark brown to black, and the root of the organism, the larval body is penetrated through the mushroom's mycelium, seems

yellowish to brown in color. Because the fungus becomes mature , it'll be consumed more than 99 % of the infested organism, effectively immunize the host.[1]

As the stroma similarly matures it begins to swell and develop perihelia. despite the fact that 400 and more Cordyceps species have been discovered, most effectively very few species have been artificially cultivated for the manufacturing of fruiting-bodies , of these species which have been artificially cultivated, simplest C. militaris has been commercially cultivated, business development has targeted on C. militaris due to its extraordinary pharmaceutical impact and brief production length Cordyceps militaris has been cultivated in liquid media for harvesting mycelia and on stable media for induction of Fruiting-bodies [2]. Even as cultures for submerged cultivation of C. militaris inoculum had been optimized massive-scale production of C. militaris fruiting bodies currently makes use of stable media together with artificial substrates or insects (e.g., the silkworm B. mori). due to the fact cultivation on insects is costly, fruiting-bodies of C. militaris are mainly cultivated on synthetic media in which brown rice is the principle component .

1.1Fermentation conditions for cordycepin production

Culture Name	Culture Condition	ph	Agitation	Temp.	Light Duration	Light Intensity	Incubation Time	Cordycepin Level	Reference
Liquid Culture	Shaking	Low	150rpm	25ºC	8 hrs	450nm	21 days	3483mg/l	Lin and Tai (2018)
Liquid culture	Shaking	NM	150rpm	25ºC	NM	NM	5 days	0.826g/l	Yang S et al (2014)
Liquid culture	Shaking	6	100rpm	25ºC	NM	NM	7 days	2.17mg/g	Xie et al (2009)
Liquid culture	Shaking	NM	220rpm	28ºC	NM	NM	7 days	2.85mg/g	Huang et al (2009)
Liquid culture	Shaking	NM	110rpm	25ºC	NM	NM	9 days	345mg/l	Xian Bing Mao
Liquid Culture	Static	6.1	NA	25ºC	NM	NM	10 days	8.57g/l	SK Das (YEAR)
Liquid Culture	Static	5.5	NA	20ºC	NM	NM	20 days	381 mg/l	Syed Amir Ashraf (2017)
Liquid culture	Shaking	7	250 rpm	80ºC	NM	NM	20 days	2560µg	Xuesong Wang (2016)
Liquid Fermentation	Shaking	4.5	100	22ºC	NM	NM	7 days	15.71g/l	Chunyan Xie et al (2009)
Liquid Fermentation	Shaking	7.5	100rpm	30ºC	NM	NM	7 days	16.72g/l	Chunyan Xie et al (2009)
Liquid fermentation	Shaking	NM	180 rpm	27ºC	NM	NM	20 days	0.21g/l	Tang Jiapeng (2015)
Liquid Fermentation	Static	NM	NA	27ºC	NM	NM	20 days	1.03 g/l	Tang Jiapeng(2015)
Liquid Static culture	Static	NM	NA	25ºC	NM	NM	35 days	2008mg/l	Kang T. C. W. C-B.-R(2014)
Liquid Surface Cultivation	Static	5.6	NM	25ºC	NM	NM	8 days	620mg/l	Nurmila Sari (2016)
lLiquid Culture	Static	4.5-7	NA	25ºC	NM	NM	30 days	591mg/l	Tuli et al (2014)
Solid Culture	Static	NM	NA	23ºC	8 hrs	150lux	21 days	6.13mg/g	Jian L.R, Li Z.F(2017)
Solid State	Static	NM	NA	25ºC	12hrs	600lux	45 days	28mg/g	Chen et al (2011)
Solid State	Static	NM	NA	23ºC	Dark	500lux	12 days	814.6mg/g	Mohd Adnan
Solid State	Shaking	NM	150rpm	23ºC	12:12	300lux	12 days	3.3mg/l	Chiang Mao J. Sci. (2014)
Solid State	Static	5.5	NA	23ºC	12hrs	500lux	12 days	814g/l	Syed Amir Ashraf(2017)
Solid State	Static	NM	NA	24ºC	White	NM	25 days	10.42mg/g	Gregori et al

					Flourescent				
Solid State	Static	5.5		23ºC	12hrs	500lux	12days	814mg/l	Syed Amir (2017)
Solid State	Static	6.1	NA	24ºC	NM	NM	25 days	10.42mg/g	Gregori (2007)
Solid State Fermentation	Static	5.5	NA	23º C	12 hrs	500lux	12 days	814mg/g	Syed Amir Ashraf
Solid state fermentation	Static	NM	NA	25ºC	NM	NM	12 days	85.78	Shih-Jeng Huang(2017)
Solid State Fermentation	Static	NM	NA	23ºC	12hrs	300lux	39 days	3.3mg/g	Chiang Mai et al (2016)
Solid State Fermentation	Static	5.5	NA	25ºC	12 hrs	300 lux	21 days	814.60mg/g	Mohd Adnan et al (2017)
Submerged	Shaking	6.3	180 rpm	25ºC	NM	NM	35 days	2.18 g/l	Tang Jiapeng (2011)
Submerged	Static	6.3	NA	25ºC	NM	NM	21 days	1.92g/l	Tang Jiapeng(2011)
Submerged	Shaking	6	220rpm	28ºC	NM	NM	7 days	3.412mg/g	Lei Huang (2009)
Submerged Cullture	Shaking	NM	25rpm	25ºC		600lux	07 days	2.3mg/g	Y.S. Chen et al
Submerged Culture	Static	5.6	NA	18ºC	NM	NM	40 days	4.45mg/g	Dong and Yao (2005)
Submerged Culture	Static	NM	NA	25ºC	NM	NM	35 days	1405.94mg/l	Ting et al (2014)
Submerged Culture	Shaking	5	150rpm	25ºC	NM	NM	7 days	215.4mg/l	Tang Jiapeng
Submerged Culture	Static	6.3	NA	25ºC	NM	NM	21 days	1.92g/l	Tang Jiapeng (2011)
Submerged Culture	Static	NM	NA	25ºC	Dark	NM	15 days	104.4mg	L.T.Hung et al
Submerged Culture	Shaking	NM	110rpm	25ºC	NM	NM	9 days	345 mg/l	Xian Bing Mao (2004)
Submerged fermentation	Shaking	NM	180rpm	27ºC	Dark	NA	21 days	7.35g/l	Tang Jiapeng et al (2014)
Submerged Surface liquid	Shaking	6	150rpm	26ºC	NM	NM	20 days	786.94mg/l	M Soltani (2016)
Submerged Surface liquid	Static	6.1	NA	25ºC	NM	NM	10 days	8.57 g/l	SK Das (2008)
Surface Liquid Cultivation	Shaking	NM	150rpm	25ºC	16 hrs	1.5 W	14 days	3483mg/l	Liang Tzung Lin (2018)
Surface Liquid cultivation	Shaking	NM	150rpm	25ºC	24 hrs	1.5 W	14 days	650mg/l	Liang Tzung Lin (2018)
YPD liquid Culture	Shaking	NM	250 rpm	22ºC	No	NA	7 days	149.5mg/l	Nachon Raethong(2018)

Table 1 The various fermentation conditions for the cordycepin production .

There are various methods for cultivation of C.militaris including solid culture, submerged culture , surface liquid culture as mentioned in the table above . Generally, C.militaris requires specific hosts and specific growth environment. hence, through artificial cultivation useful and potent cellular and extracellular substances could be obtained .

Tang japing and coworker's (2016) grow *C. sinensis (Berk)* at ph 5, 25°C and a yield of 215.4 mg/l cordycepin was obtained after 7 days of incubation under submerged shaking conditions. It was noted that *Cordyceps sinensis* CS1197 when grown under liquid static conditions with ph 4.5-7, 25°C could produce 591 mg/l cordycepin after 30 days of incubation (tuli et al (2014)). 3.3mg/l cordycepin was obtained after 12 days of incubation by Chiang Mai et al. 2014; with the strain C. *militaris* 34164 conditions maintaining temperature 23°C light of intensity 300 lux for 24 hrs under solid state fermentation. However a maximum of 1.92 g/l cordycepin was obtained when *C. sinensis (Berk)* at ph 6.3, 22°C after 21 days of incubation in submerged static conditions. Chen and group reported 2.3±0.5 cordycepin production after 7 days of incubation at 25°C

intensity of light 600 ±20 lux for 40 hrs under submerged shaking conditions. Interestingly *C. militaris* could produce 814g/l of cordycepin after 12 days of incubation under solid static fermentation conditions with ph 5.5, 23°C light of intensity 500x for 12 hrs. (Sayed amir et al 2014). Moreover a yield of 8.57g/l cordycepin was obtained when *C. militaris* NBRC 9787 at ph 6.1, 25°C was incubated for 10 days under surface liquid culture conditions by Sk das and group (2008). It was found that *C. militaris* CM11 when grown under solid static fermentation at temperature 24°C in presence of white flourecent light and incubated for 25 days produced 10.42 mg/g cordycepin (Gregori et al). L.T. Hung and group reported 104.46 ± 45.45 mg/l cordycepin production after 15 days of incubation under dark conditions at 25°C with the strain *C. militaris* BCC2790. Chunyan XIE, Gaixia et al grow *C. militaris* CM14 at ph 4.5, 22°C and a yield of 15.71 g/l cordycepin was obtained after 7 days of incubation under liquid shaking fermentation whereas Amir and coworkers, 2017 when grew *Cordyceps militaris* CGMCC2459 at ph-6.3, 25°C, yield of 814 mg/L cordycepin was obtained after 35 days of incubation. However, in another study 786.94m/L cordycepin production was obtained when *Cordyceps militaris* DSMZ 23612 was grown at 26°C, ph-6, agitation- 150 in shaking condition with an incubation period of 20 days (M. Solatani 2016 et al). It was noted that 2.18 g/L and 1.92 g/L cordycepin was produced in shaking and static conditions respectively keeping other parameters same as ph-6.3, 25°C, with an incubation time of 35days and 21 days respectively.(Tang Jiapeg 2011 et al). Interestingly, *Cordyceps militaris* BCC2824 would produce 0.21 g/L and 1.03g/L of cordycepin under shaking and static conditions at 27°C, with incubation time of 20 days.(Tang Jiapeg 2015 et al). It was also noted that under static solid state condition, 10.42mg/g of cordycepin was produced at 24°C in 25 days. 8.54g/L cordycepin was obtained using *Cordyceps militaris* NBRC 9787 under the solid state static conditions, maintaining 24°, ph-6.1 with an incubation time of 25 days (Gregori 2007 et al).

1.3 Therapeutic potential of cordycepin

- **Antifatigue -**Cordyceps has many applications in health improvement and medicinal uses; the best known is the relief of fatigue .Today, people living in mountains consumes Cordyceps it provides them energy and helps them to deal with altitude sickness (Drel and Sybirna, 2006). In a placebo-controlled clinical study of elderly patients with chronic fatigue, results showed that most treated with C*ordyceps sinensis* reported significant clinical improvement in the areas of fatigue, cold intolerance, dizziness and amnesia, while no improvement was reported in the placebo group. The significant results approved that Cordyceps group leads to significant increase in oxygen and high energy output over the placebo group after 6weeks on Cordyceps. The most efficient test to determine whether a compound can increase energy output and reduces fatigue is mouse swim test. Hot water extraction of mycelium of Cordyceps

mainly contains carbohydrates (78 %) was administered to mice to check the swimming capacity. Cordyceps was able to prolong the swimming time (75-90 min) of test groups compared to control group which indicates that hot water extraction of Cordyceps helps in recovery of exhaustion by lessening of fatigue (Koh et al., 2003). Incredible performance by Chinese women athletes at Chinese national games in Beijing in September 1993 astounded the world of international track and field.

- **Kidney treatment-** Patients suffering from chronic renal failure or reduced kidney function often suffer from hypertension, proteinuria and anemia. The treatment of these patients for on month with Cordyceps, 15% reduction in blood pressure was observed also leads to increase in superoxide dismutase (SOD) and urinary protein was also reduced. The increase in SOD leads to reduced oxidative cellular damage(Jiang and Gao, 1995) .Cyclosporine A (CsA) it cannot be used for the long term treatment of kidney allograft recipients due to severe side effects . when patients having renal transplant are treated with Cordyceps it cause two beneficial effects – 1) least incidences of complications in the treatment group, (2) no significant difference in the serum level of IL-2 in two groups and the serum level of IL-10 in treatment group (Ding et al., 2009, 2011).The study contributed that Cordyceps can be used in combinations with a low dose of CsA in the long term treatment of kidney transplant patients in several acute and chronic experiments Cordyceps showed to protect the kidney from cyclosporine A nephrotoxicity (CsA-Nx).Rats having renal tubular injury showed best results of improvements after treatment, many of the Cordyceps kidney enhancing potential is due to the ability to increase 17-hydroxy-corticosteroid and 17-ketosteroid levels (Zhou et al 1998). Kidney failure can also lead to failure of several another organs. Further studies included 57 patients with gentamicin induced kidney damage were treated with 4.5 g of Cordyceps per day (Guan et al 1992). Cordyceps improved normal kidney function (89%) as compared to control group (45%) after six days, indicating CS effectively shortens the time to recovery significantly.
- **Anti-diabetic:** Cordycepin, a nucleoside derivative isolated from Cordyceps, has been reported to exert anti-inflammatory, antitumor, anti-diabetic and Reno protective effects. It has been studied that *cordyceps militaris* reduces oxidative stress produced due to high glucose concentrations(Mao, X.B et al 2005). The active dose taken was 25 µL in HUVECs cell. Another study showed that Fractions of *C. militaris* as CMESS and Cordycepin was tested on Mice which acted as anti- diabetic drug when an active dose of 50 and 0.2 mg/kg, respectively was given(Cunningham et al 2000). Crude extract and polysaccharide rich fraction when given to rat, in 10 mg/kg of polysaccharide and 100 mg/kg body weight of crude extract, helped in lowering sugar levels.

- **Immunomodulator -**The human immune system protects the body from infectious microorganisms. It does so by differentiating in self and non-self. There is varying line of defense in the human immune system, namely the first line of defense also known as innate immunity or non-specific immunity, and second line of defense known as adaptive immunity. The cells of the innate immunity include granulocytes (eosinophils, basophils and neutrophils), mast cells, natural killer cell and macrophages. Adaptive immunity is further divided into humoral immunity which involves B cells and Cell-mediated immunity which involves the T cells.Immunopotentiating drugs are used to restore the functioning of immune system to normal and to prevent the reoccurring of life-threatening diseases. Immunosuppressants are used to control auto immune diseases and reduce inflammation after tissue damage, and to prevent organ rejection after transplantation. Cordyceps are used as both Immunopotentiating drugs and immunosuppressant (Li S. P, 2004) .Some of the accounted examples of C. militaris as an immunomodulator are : Fruiting bodies of *C. militaris* water extract significantly upregulated IL-18 gene expression, an inducer of IFN-γ in T cells and NK cells, in C57BL/6 mouse brain and liver in vivo and in RAW264.7 cells in vitro. A C. *militaris* water extract induced phenotypic and functional maturation of dendritic cells, which then initiated T-cell responses against microbial pathogens and tumors (Kim G. Y, 2006).The latest reports show that Cordyceps have an anti-rejection effect in the experimental research of organ transplantation. For example, Guan and Yu studied the prevention of rejection of transplanted kidney by artificial cultured *Cordyceps* in rats; the results showed that Cordyceps powder can markedly prolong rat renal allograft survival (Professor Xuanwei Zhou, 2010).The anti-inflammatory and immune- promoting effects can potentially facilitate the treatments of other diseases such as arthritis, HIV and Crohn's disease. Since this fungi is edible and thus can be a food additive or supplement will play a key role in the prevention and cure of various ailments caused by metabolic disorder or infections (Aarti Mehra, 2017).

2. The main objectives of the studies were:

1. Optimization of cordycepin extraction conditions
2. Optimization of cultivation conditions (Submerged fermentation, Solid state fermentation and liquid static fermentation) on production of cordycepin from *C. militaris*.
3. Evaluation of the effect of ph in solid, liquid shaking and liquid static conditions on production of cordycepin from *C. militaris*.

4. Evaluation of the effect of substrate in solid and shaking conditions on production of cordycepin from *C. militaris.*

3. MATERIALS AND METHODS

Raw materials: Potato dextrose broth, agar, peptone were collected from Hi Media Laboratories Pvt. Ltd. India. Sucrose, magnesium nitrate hexahydrate, acetonitrile, methanol was collected from Merck Life Science Pvt. Ltd. Mumbai, India. Potassium sulphate and acetone was collected from Thermo Fisher Scientific India Pvt. Ltd. Sodium Phosphate Dibasic Anhydrous was collected from Sisco Research Laboratories Pvt. Ltd. India. Ethanol was collected from EMD Millipore, Germany. Brown rice, wheat bran and honey were purchased from local stores.

Culture maintenance and storage: The isolate of C.militaris was collected from SVI Analytica. The micro-organism was maintained on Potato dextrose agar plates. Culture was revived using a 8mm disc of C.militaris and the plates were left for incubation for 15 days at 25ºC at 90% humidity and then stored at 4ºC. Radial growth of the plates was observed on a daily basis.

Growth study

Culture plates were prepared potato dextrose agar having following composition 5 ml water 1.2 gm PDB and 1 gm agar was autoclaved and inoculated with already prepared inoculum of C. militaris, mycelial growth was observed each day after the day of inoculation.

3.1 PRODUCTION MEDIA AND INNOCULATION

Potato dextrose broth was prepared and inoculated with already prepared disc culture. This was allowed to grow for 10 days. After 10 days, mat was removed. This was crushed with PDB using mortar pestle. It was preserved for further inoculation.

3.1.1 Optimization of cultivation conditions

The basal medium composition for the liquid static culture was as follows: sucrose 20 g/L; peptone 20 g/L; wheat bran 25 g/L; brown rice 25g/L; K2SO4 0.2g/L; MgNO3 0.2 g/L; Na2HPO4 0.2 g/L; honey 250 µl; PDB 24 g/L. Four flasks were prepared with a working volume of 50 ml each.

The basal medium composition for the liquid shaking culture was as follows: sucrose 20 g/L; peptone 20 g/L; wheat bran 25 g/L; brown rice 25g/L; K2SO4 0.2g/L; $MgNO_3$ 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; PDB 24 g/L. Four flasks were prepared with a working volume of 50 ml each.

The solid static culture experiments were performed in 1000mL glass jars (inner diameter 110 mm, height 150 mm) containing basal medium concentration as follows: sucrose 20 g/L; peptone 20 g/L; K_2SO_4 0.2g/L; $MgNO_3$ 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; brown rice 300 g/L. A working volume of 50ml was used for preparation of each jars. The culture was incubated at 25∘C without moving for 15 days and samples were collected at the end of the fermentation from the glass jars for analysing biomass dry weight and cordycepin production.

3.1.2Effect of ph

The basal medium composition for the liquid static culture was as follows: sucrose 20 g/L; peptone 20 g/L; wheat bran 25 g/L; brown rice 25g/L; K_2SO_4 0.2g/L; M_gNO_3 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; PDB 24 g/L. Four flasks were prepared with a working volume of 50 ml each. The pH was adjusted to 5, 6, 7 and 8, one flask for each ph followed by autoclaving for 30min at 121°C. The culture was incubated at 25ºC without moving for 15 days.

The basal medium composition for the liquid shaking culture was as follows: sucrose 20 g/L; peptone 20 g/L; wheat bran 25 g/L; brown rice 25g/L; K_2SO_4 0.2g/L; $MgNO_3$ 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; PDB 24 g/L. Four flasks were prepared with a working volume of 50 ml each. The pH was adjusted to 5, 6, 7 and 8, one flask for each ph followed by autoclaving for 30min at 121°C. The culture was incubated for 15 days on a rotary shaker at 150 rpm.

The solid static culture experiments were performed in 1000mL glass jars (inner diameter 110 mm, height 150 mm) containing basal medium concentration as follows: sucrose 20 g/L; peptone 20 g/L; K_2SO_4 0.2g/L; $MgNO_3$ 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; brown rice 300 g/L. A working volume of 50ml was used for preparation of each jar. The pH was adjusted to 5, 6, 7 and 8, one jar for each ph followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C without moving for 15 days and samples were collected at the end of the fermentation from the glass jars for analyzing biomass dry weight and cordycepin production.

3.1.3 Effect of different substrates

The basal medium composition for the liquid shaking culture was as followssucrose 1 gm/50ml; peptone 1g/50ml; K_2SO_4 0.01g/50ml; $MgNO_3$ 0.01g/50ml; Na_2HPO_4 0.01g/50ml; honey 250 µl; PDB 1.2g/50ml. six

flasks were prepared with each flask containing different substrate,i.e. wheat, corn, bajra, sprouts, rice and brown rice respectively. The pH was not adjusted followed by autoclaving for 30min on the 121◦C. The culture was incubated at 25ºC without moving for 15 days.

The solid static culture basal medium concentration was as follows: sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 25 gm of substrate was added to each jar keeping the substrate different for each jar. The pH was not adjusted followed by autoclaving for 30min on the 121◦C. The culture was incubated at 25◦C without moving for 15 days.

3.1.4 Effect of light

Three different light conditions were taken into consideration as mentioned below

- light for 8 hours in a day
- light for 16 hours in a day
- dark condition

Effect of light for 8 hours in a day

The solid static culture experiments were performed in 1000mL glass jars (inner diameter 110 mm, height 150 mm) containing basal medium concentration as follows: sucrose 20 g/L; peptone 20 g/L; K_2SO_4 0.2g/L; $MgNO_3$ 0.2 g/L; Na_2HPO_4 0.2 g/L; honey 250 µl; brown rice 300 g/L. A working volume of 50ml was used for preparation of each jar. The pH was adjusted to 6followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C without moving for 15 days provided 8 hours of light in a day and samples were collected at the end of the fermentation from the glass jars for analyzing biomass dry weight and cordycepin production.

Effect of light for 16 hours in a day

The solid static culture basal medium concentration was as follows: sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 25 gm of substrate(brown rice) was added to each jar , followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C without moving for 15 days provided 16 hours of light in a day .

Effect of dark condition

The solid static culture basal medium concentration was as follows: sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 25 gm of substrate (brown rice) was

added to each jar, followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C without moving for 15 days in presence of no light.

3.1.5 Effect of substrate concentration

Two different substrate concentrations were considered 15 gm and 25 gm.

Substrate concentration 15 gm

The solid static culture basal medium concentration was as follows: sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 15 gm of substrate was added to each jar keeping the substrate different for each jar, followed by autoclaving for 30min on at 121°C. The culture was incubated at 25°C without moving for 15 days.

Substrate concentration 25 gm

The solid static culture basal medium concentration was as follows: sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 25 gm of substrate was added to each jar , followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C without moving for 15 days.

3.1.6 Effect of time course

Fourteen jars were prepared as follows solid static culture basal medium concentration was sucrose 1gm; peptone 1gm; K_2SO_4 0.01gm; $MgNO_3$ 0.01gm; Na_2HPO_4 0.01gm; honey 250 µl and 50ml water. 25 gm of substrate (brown rice) was added to each jar , followed by autoclaving for 30min at 121°C. The culture was incubated at 25°C and every three day two jars were removed and subjected to drying and extraction process, results were observed using HPLC.

Analytical method: Samples collected at 15 days from the flasks were centrifuged at 3800 rpm for 20min. The mycelium at the bottom of tubes was dried to a constant dry weight at 55∘C. For analysis of extracellular cordycepin, the resulting culture filtrate was obtained by centrifugation at 3800 rpm for 20min. The supernatant was filtered through filter syringe.

The filtrate was analyzed by HPLC (EWAI 5510) equipped with UV detector and a reverse phase C-8 column. Accurate quantities of cordycepin (Sigma, USA) were dissolved in acetonitrile, to give various concentrations for calibration. The mobile phase was acetonitrile, which was dissolved in acetonitrile/distilled water (70:30).

Elution was performed at a flow rate of 1.50 mL/min with column temperature at 40∘C and UV wavelength of 210 nm. Mean values were computed from triplicate samples.

- **CHNS ANALYSIS**

The determination of Carbon, Hydrogen, Nitrogen and Sulphur content is carried out by ECS 4010 CHNSO ANALYZER. Solid and samples, weighed in tin capsules, are introduced into a vertical quartz reactor heated at a temperature of 1250°C of left furnace with a constant flow of helium stream. The combustion gas mixture is driven through an tungsten oxide zone to achieve a complete quantitative oxidation followed by a reduction step in a copper N_2 ,CO_2,H_2O,SO_2 to reduce nitrogen oxides and sulfuric anhydride to nitrogen and sulfurous anhydride. The resulting four components N_2, CO_2 , H_2O and SO_2 are separated in a gas chromatographic column kept at temperature 80°C and detected by a thermo conductivity detector. The resulting signals, proportional to the amount of eluted gases, are analysed by EAS32, Windows TM based software package for instrument control, data acquisition and report generation which provides the sample elemental composition report.

- **UV- VIS SPECTROPHOTOMETER ANALYSIS**

  UV-Vis spectra were recorded using as a light source versatile lamp optimized for the visible-near infrared Vis-NIR (360–2000 nm), Ocean Optics, HL-2000 (tungsten light source). To collect UVVis spectra, the detector (miniature fiber optic spectrometer) was used. Before measurement, Ninhydrin was added to the samples and were diluted one hundred timesfor accurate protein estimation .

3.2EXTRACTION METHODOLOGY

1. **PREPARATION OF MYCOSUBSTRATE**: The fungal mycelia that has been stored for 90 days was collected, dried and grounded to a fine powder form. The powder was sieved and collected in an air tight container for further analysis.
2. **SELECTION OF EXTRACTION SOLVENT:** Five different solvents namely acetone, acetonitrile, ethanol, methanol and water were used for extraction. Instrument used for the extraction process was Soxhlet appararus. Amount of substrate used was 2mg. The temperature, time and volume of solvent were set at 50ºC, 60 minutes and 30 ml. The substrate to solvent ratio was 1:15. After extraction, HPLC

was performed to check the cordycepin concentration and by the results, water came out to be the best extraction solvent.

3. **OPTIMIZATION OF CORDYCEPIN EXTRACTION**: For the optimization of cordycepin extraction from mycosubstrate, a design was prepared using BOX- BEHNKEN DESIGN of Response Surface Methodology with three parameters; i.e; extraction time, temperature and volume of extraction solvent.

4. Observation And Results

4.1 GROWTH OBSERVATION

Cultures were inoculated with inoculums of mycosubstrate and growths of the pates were measured till ten days and growth curve was plotted.

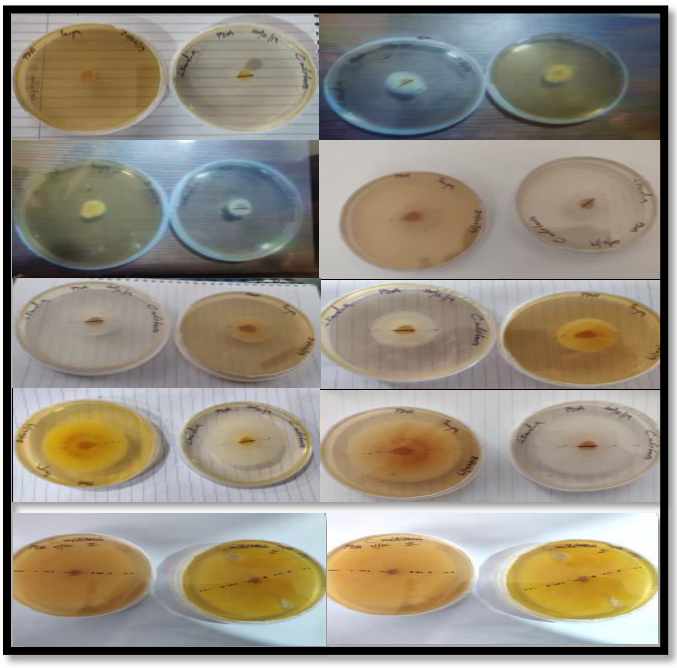

Figure 1: Growth observation of 10 days

Days	Growth Measured
1	10.9
2	13.5
3	17.5
4	24.5
5	28.5
6	35.5
7	41.5
8	48.5
9	52.5
10	56.5

Table 1: Growth measurements

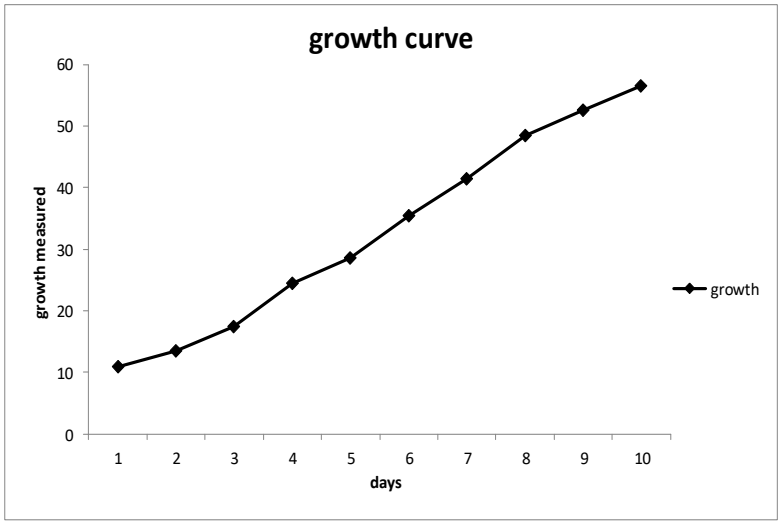

Figure 2: Growth curve

4.2 SELECTION OF SOLVENT

Results were obtained by carrying out the extracted samples on high performance liquid chromatography. Different solvents were used. Maximum concentration of cordycepin was obtained with water as solvent and minimum was obtained with acetonitrile.

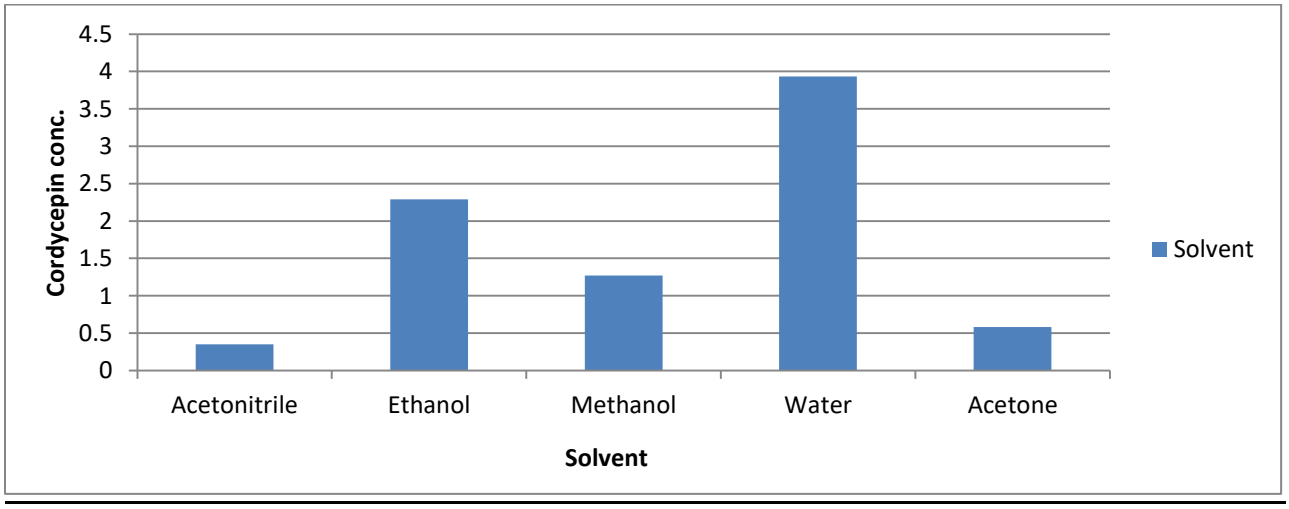

Figure 3: Effect of different types of extracting solvents

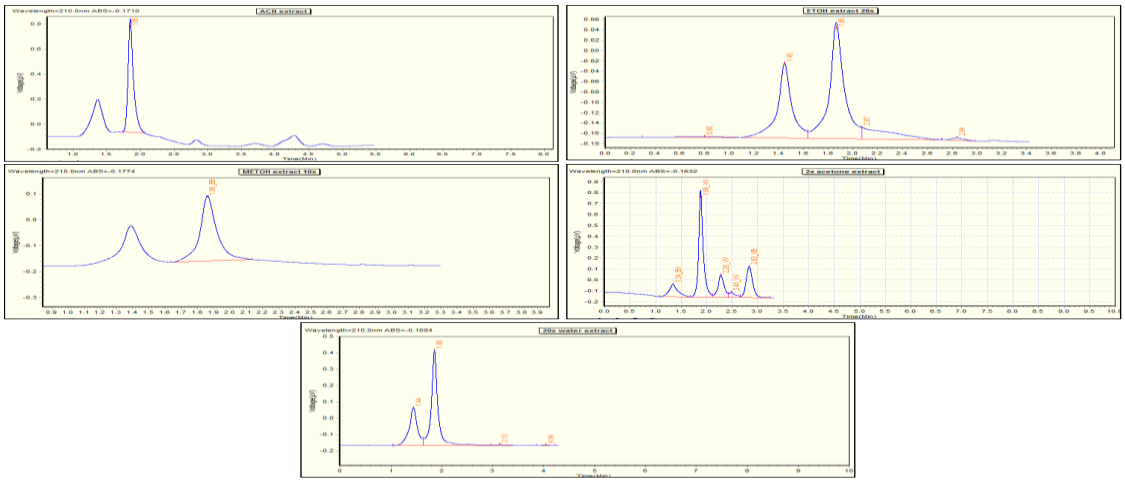

Figure 4: HPLC chromatograms to check effect of different solvent

Run	Factor A: Temperature (°C)	Factor B: Time (min)	Factor C: Volume (ml)	Response: Cordycepin (mg/g)
1	50	45	15	0.450
2	65	60	15	1.813
3	50	60	20	6.320
4	65	45	20	4.220
5	35	45	20	4.580
6	50	45	15	0.450
7	65	45	10	0.007
8	50	45	15	0.450
9	35	30	15	0.280
10	50	30	10	0.060
11	35	45	10	0.007
12	50	30	20	1.510
13	35	60	15	2.690
14	65	30	15	0.155
15	50	60	10	0.003
16	50	45	15	0.450
17	50	45	15	0.450

Table 2:Box-Behnken design of experiment of Response Surface Methodology (Design Expert ver 5.0) for optimum extraction of cordycepin.

ANOVA test was used in order to determine the significance of the variables to the response. The result obtained from ANOVA is shown in Table 3 presented belowThree investigated variables, viz., temperature, time and volume were embarked as the most effective ones influencing the yield of enzyme with their significant *p* values where the values of "Prob > *F*" less than 0.05 specify model terms are significant, while remaining components show *p* values (0.1) which are above the significant level; hence, those components are considered to be insignificant.

The main effect of medium components, standard analysis of variance (ANOVA), regression coefficient, *F* values, and *p* values of variables examined in this study, and are illustrated in Table 3. The model *F* value 58.8225 implies that the model is significant and there is only 0.01% chance that this large value could occur due to noise; in addition, the pre-determined R^2 0.995 was in reasonable agreement with the adjusted $R^2$0990. The adequate exactitude measures signal-to-noise ratio. A ratio greater than 4 is desirable and 12:68 ratios indicated an adequate signal; thus, this model can be used to navigate the design space. The final equation was derived in terms of actual factors which revealed the yield of enzyme as function of independent variables represented in Table 3.The primary linear empirical model in terms of coded variables and actual one is presented as an equation as follows:

Quadratic equation obtained from model for extraction of cordycepin using coding values= +0.45 -0.17A+1.10B+2.07C+0.51A2+0.28B2+1.25C2-0.19AB-0.090AC+1.22BC

Quadratic equation obtained from model for extraction of cordycepin using actual values=19.02-0.18A-0.24B-1.75C+0.002255A2+0.001232B2+0.05C2-0.0008356AB-0.0012AC+0.016BC

Model Sum of square value	58.8225
F value	190.653
Prob > F	< 0.0001
R-Squared value	0.995937
Adj R-Squared value	0.990713

Pred R-Squared value	0.934993
Adeq Precision	46.2783
C.V.	13.1726
PRESS	3.8395

Table 3:ANOVA for Response surface quadratic model

Using PB experimental design, only effective critical components affecting cordycepin production were identified, but it does not examine the interactive effects between selected critical components which were further studied by employing central composite design (CCRD) of RSM.

Factor	**Coefficient Estimate**	**DF**	**Standard Error**	**Prob > |t|**
Intercept	0.45	1	0.0828025	
A-Temperature	-0.17025	1	0.0654611	0.0354
B-Time	1.10262	1	0.0654611	< 0.0001
C-Volume	2.06912	1	0.0654611	< 0.0001
A^2	0.507375	1	0.0902319	<0.0010
B^2	0.277125	1	0.0902319	<0.0500
C^2	1.24613	1	0.0902319	< 0.0001
BC	1.21675	1	0.092576	< 0.0001

Table 4: Significance of all the factors used in the quadratic model

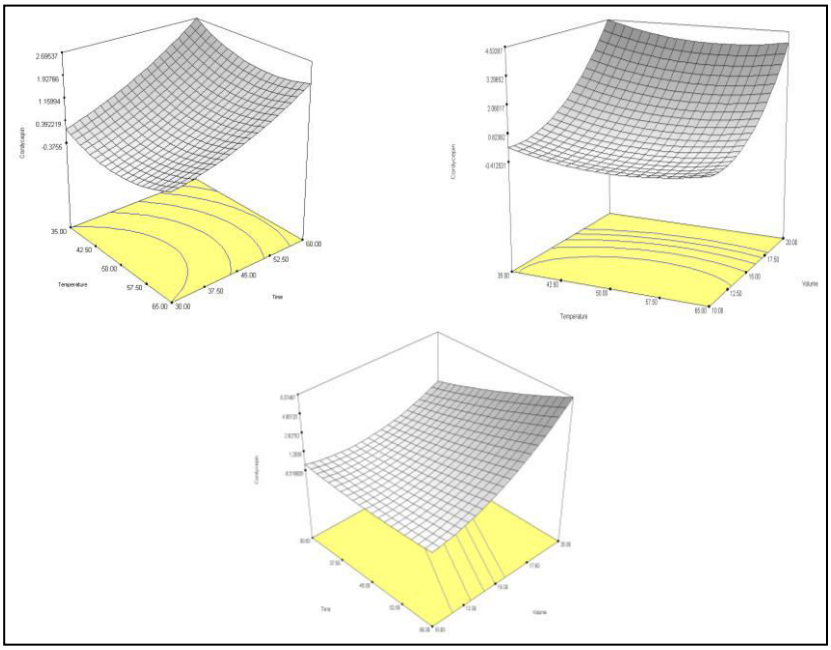

Figure 3: Response surface plots between Temperature and Time (A), Temperature and volume (B) and Volume and Time (C), while the other factor is kept constant at its '0' level.

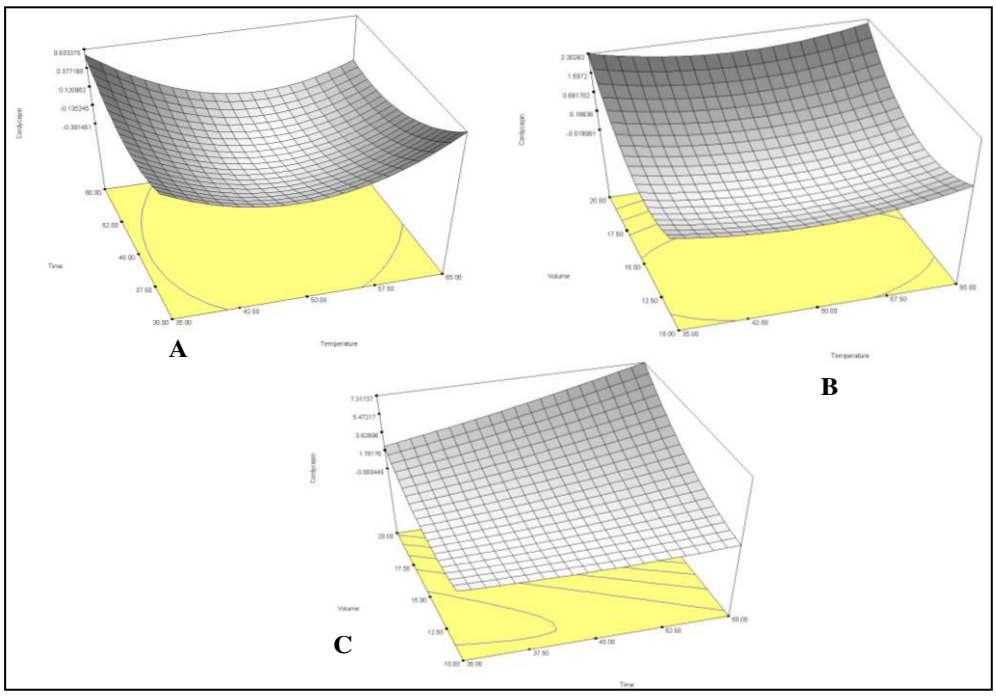

Figure 4: Response surface plots between Temperature and Time (A), Temperature and volume (B) and Volume and Time (C), while the other factor is kept constant at its '-1' level.

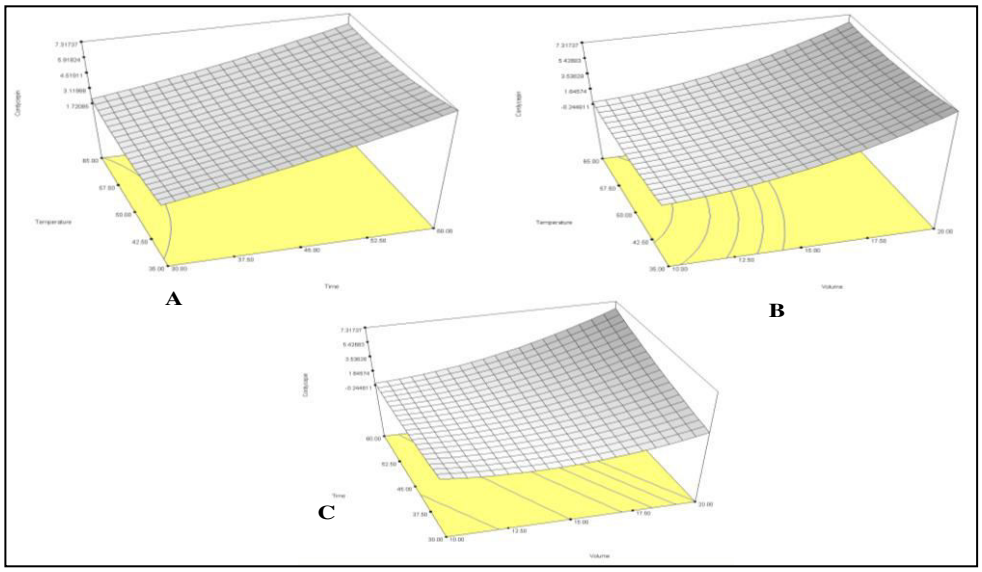

Figure 5: Response surface plots between Temperature and Time (A), Temperature and volume (B) and Volume and Time (C), while the other factor is kept constant at its '+1' level.

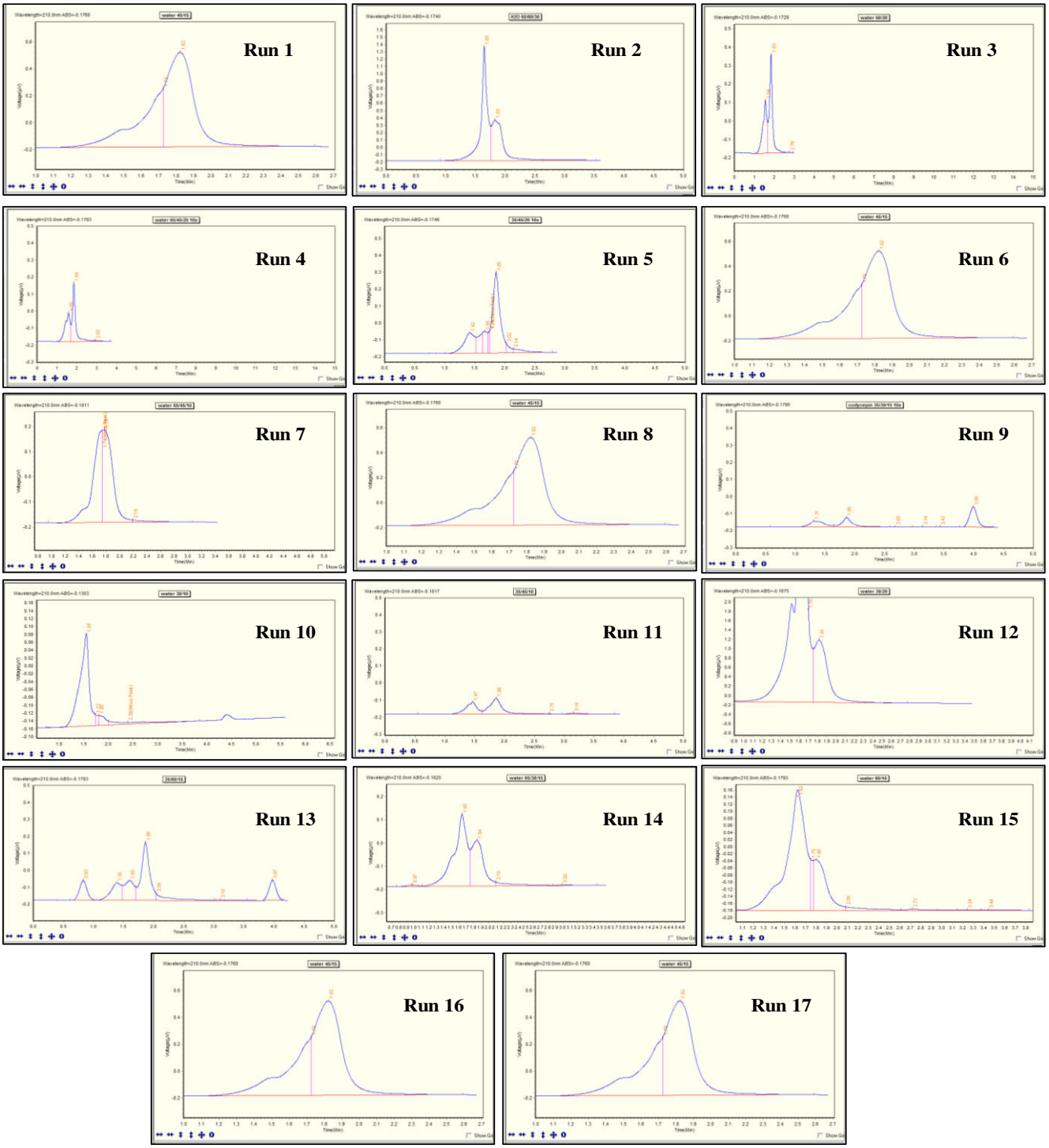

Figure 6: HPLC Chromatograms of various experimental runs of design of experiment for extraction of cordycepin.

4.3 OPTIMIZATION OF CULTIVATION CONDITIONS:

After preparation of the broth media, results were obtained by carrying out the extracted samples on high performance liquid chromatography. Three conditions were taken, solid fermentation, liquid shaking fermentation and submerged fermentation.

Broth		
Static		**Concentration**
	ph 5	2.229652
	ph6	2.1898
	ph7	2.914935
	ph8	3.410833
Shaking		**Concentration**
	ph 5	11.69624
	ph 6	1.99553
	ph 7	5.852427
	ph 8	1.596043

Table 5: Results obtained after samples extracted from broth were performed on HPLC

Another set of experiment was carried out where static liquid biomass extraction was performed on HPLC. 4 different flasks were made which were set on different ph values (ph 5, ph 6, ph 7 and ph 8). Flasks were kept in static condition only.

Static Liquid Biomass		
	Concentration	
Static	ph 5	9.860441
50/45/30	ph 6	9.988281
	ph 7	10.82162
	ph 8	9.411977

Table 6: Results obtained after LSF samples extracted from broth were performed on HPLC

Another set of experiment was carried out where static solid biomass extraction was performed on HPLC. 4 different flasks were made which were set on different ph values (ph 5, ph 6, ph 7 and ph 8). Flasks were kept in static condition only.

Static Solid Biomass		
	Concentration	
Static	ph 5	10.45713
50/45/30	ph 6	10.2323
	ph 7	11.26841
	ph 8	10.80277

Table 7: Results obtained after samples extracted from broth were performed on HPLC

Analyte	SmF	LSF		SSF
		Mat	Broth	
Cordycepin	61.52 mg	46.92 mg	19.97 mg	66.50mg
Protein	149.7			

Table 8: Effect of cultivation condition

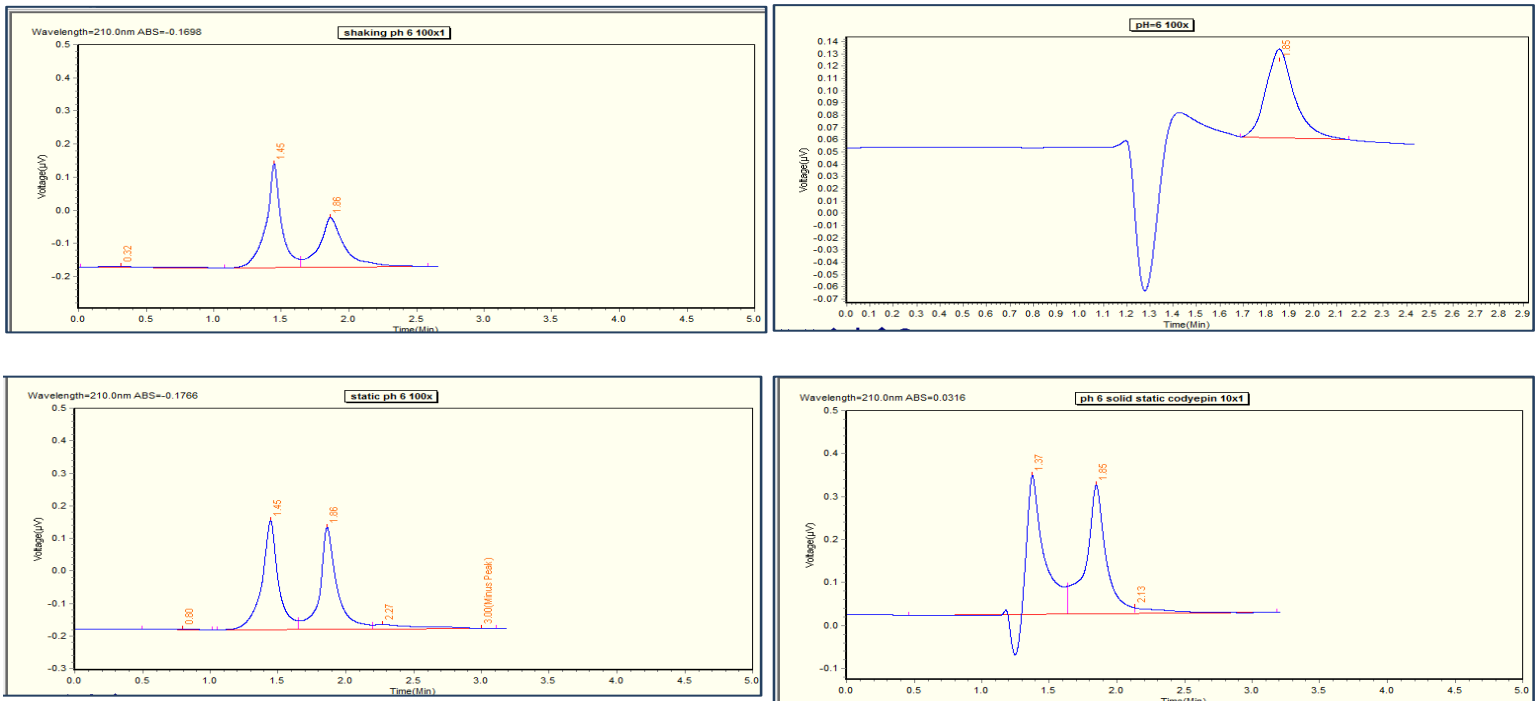

Figure 7: HPLC chromatograms for cordycepin quantification in different cultivation conditions

4.4 EFFECT OF pH

Results were obtained by carrying out the extracted samples on high performance liquid chromatography. In liquid static culture, the cordycepin content was not that good at neutral ph, but it was good at ph 5. In liquid shaking culture, best results came out with an amount of 175.44mg of cordycepin whereas least amount of cordycepin was obtained at ph 8 (35.43mg/37ml). Under solid state fermentation conditions, 73.24mg of cordycepin was attained at ph 7 whereas 66.5mg of cordycepin was obtained at ph 6. The cordycepin amount achieved under different conditions at different ph is mentioned in the table below:

table below:

Effect of pH on Liquid Static					
Ph	**Broth**		**Biomass**		
	Conc. (mg/g)	**Total (mg/20ml)**	**Conc. (mg/g)**	**Weight(grams)**	**Total (mg/49ml)**
5	8.91	44.59	9.86	1.97	19.52
6	9.38	46.92	9.98	2.26	19.97
7	8.74	43.72	10.82	1.71	18.5
8	9.49	47.46	9.41	1.83	17.31

Table 9: Effect of pH in SmF

Effect of pH on Liquid Shaking		
pH	**Conc. (mg/g)**	**Total amount(mg/37ml)**
5	35.08	175.44
6	12.3	61.52
7	17.55	87.78
8	7.08	35.43

Table 10: Effect of pH in LSF

Effect of pH on Solid State Fermentation				
pH	**Amount (mg/g)**	**Protein**	**Total Amount**	**Conc. (mg/49ml)**
5	5.22	2.50%	67.97	10.45
6	5.11	2.50%	66.5	10.23
7	5.63	2.40%	73.24	11.26
8	5.4	2.30%	70.21	10.8

Table 11: Effect of pH in SSF

pH	**Cordycepin (mg)**				**Protein**		
	SmF	**LSF**		**SSF**	**SmF**	**LSF**	**SSF**
		Mat	**Broth**				
5	175.44	19.52	44.59	67.97	385.2		
6	61.52	19.97	46.92	66.5	149.7		
7	17.55	18.5	43.72	73.24	378.7		
8	7.08	17.31	47.46	70.21	175.3		

Table 12: Effect of pH on cordycepin production in different cultivation conditions

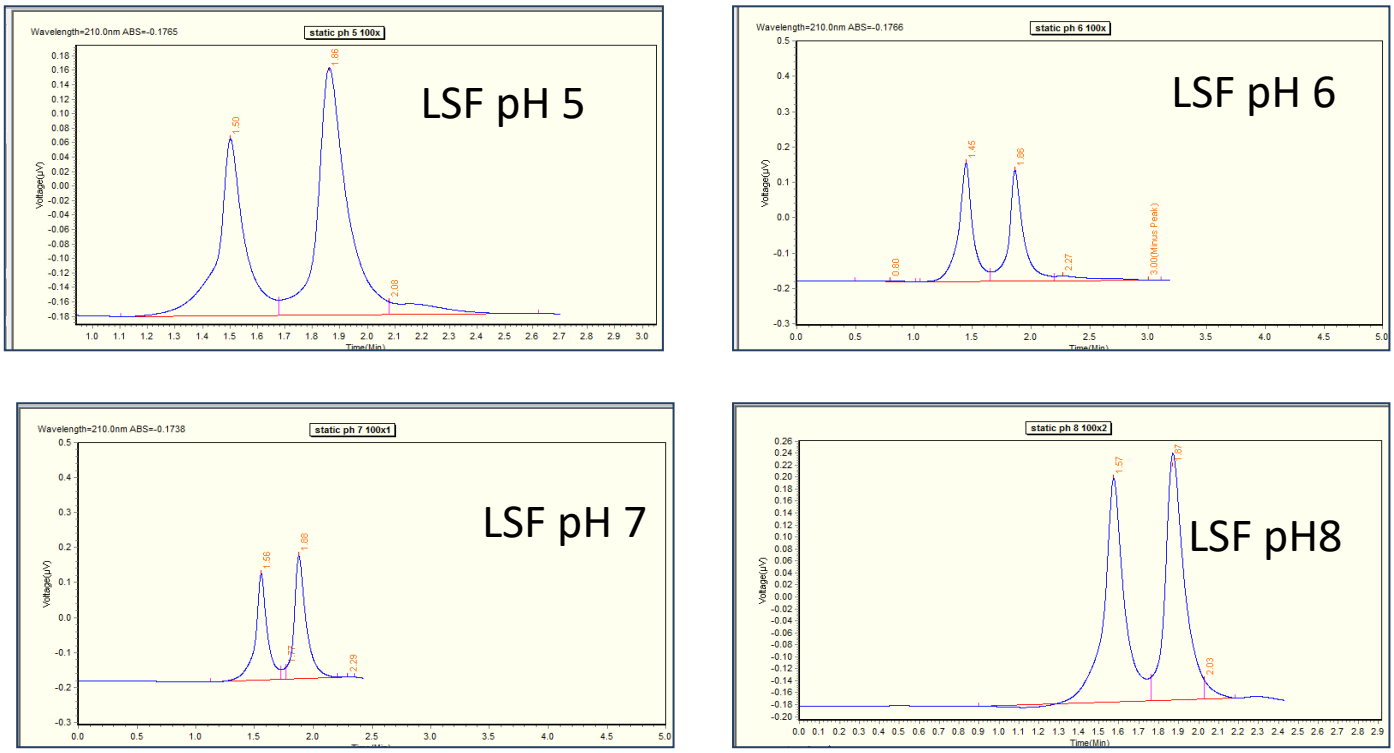

Figure 8: HPLC chromatograms for cordycepin quantification in SmF conditions (Liquid broth)

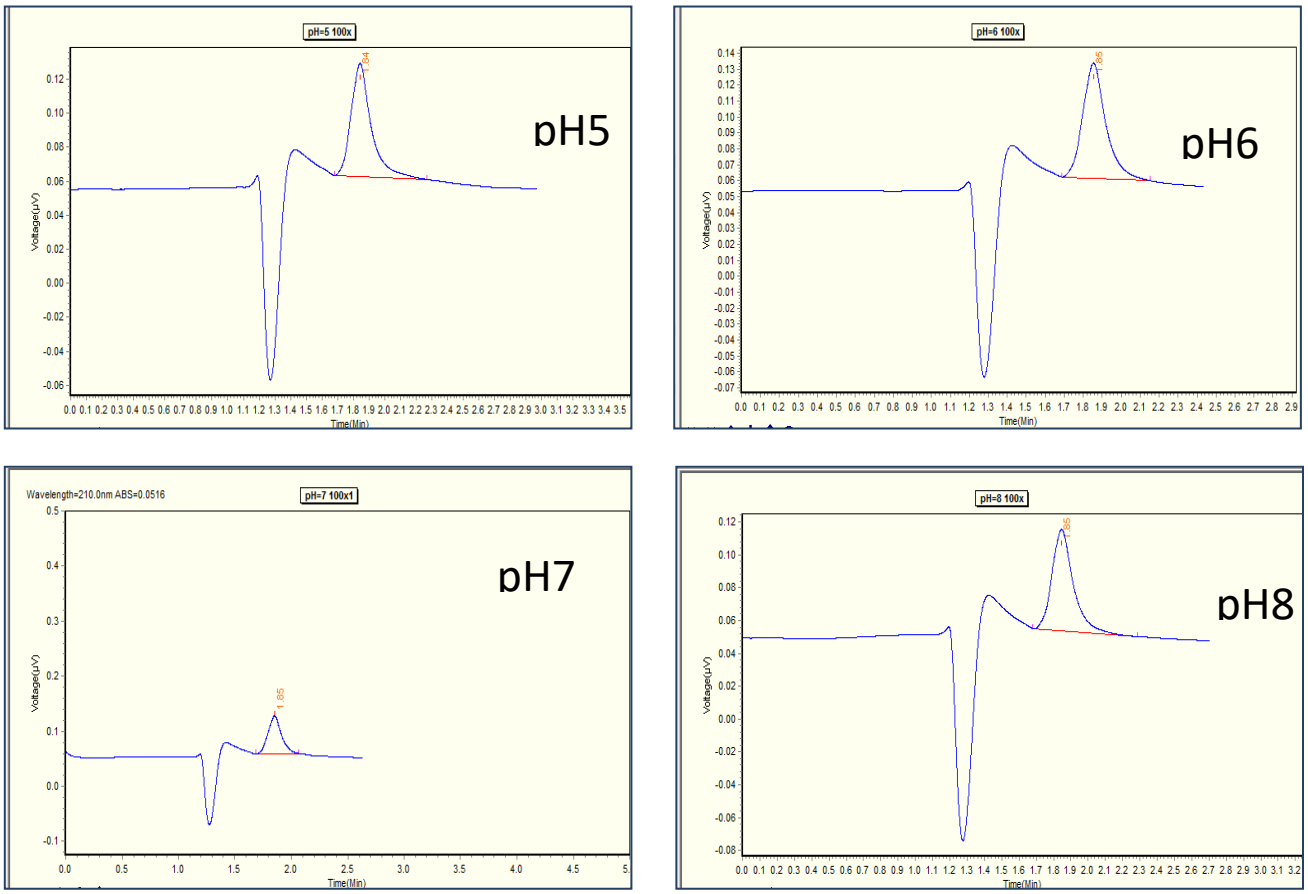

Figure 9: HPLC chromatograms for cordycepin quantification in SmF conditions (Liquid Biomass)

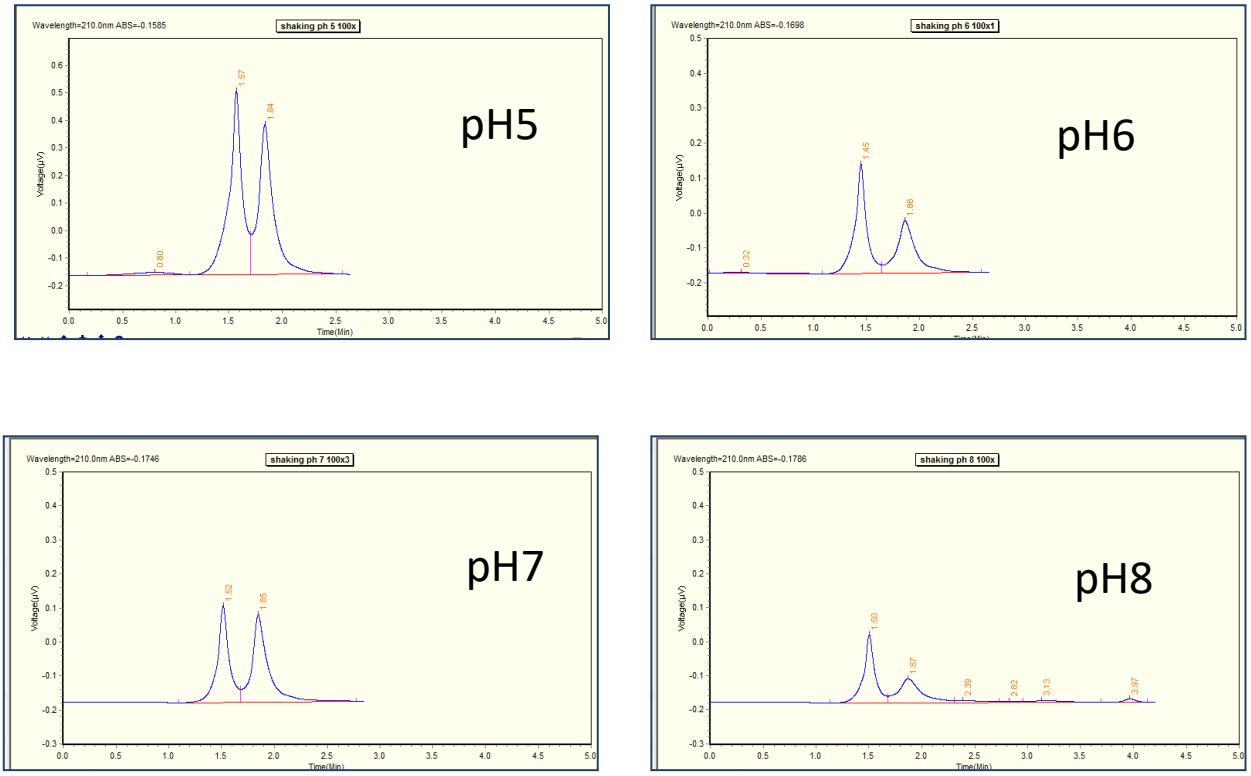

Figure 10: HPLC chromatograms for cordycepin quantification in liquid shaking condition

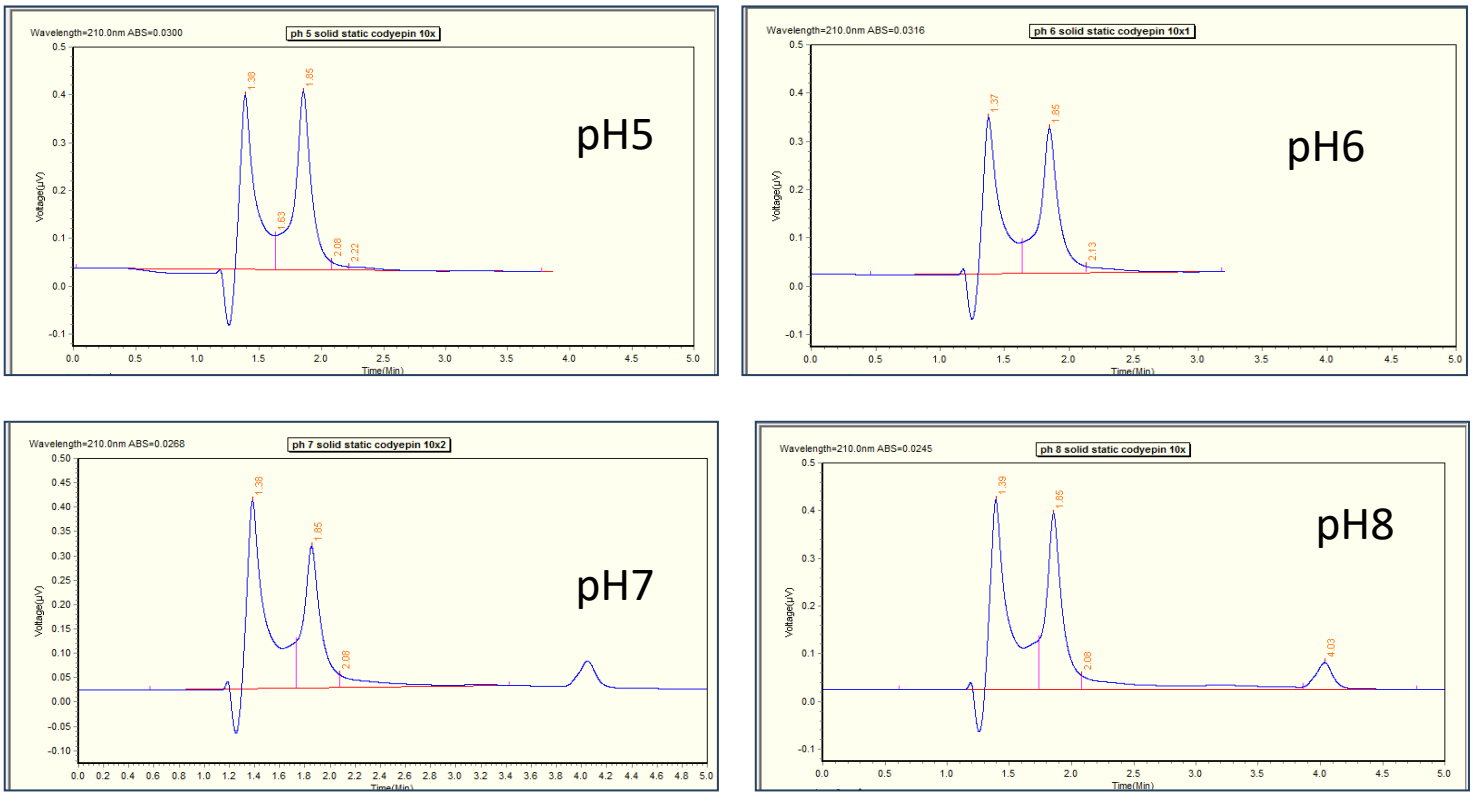

Figure 11: HPLC chromatograms for cordycepin quantification in Solid Static condition

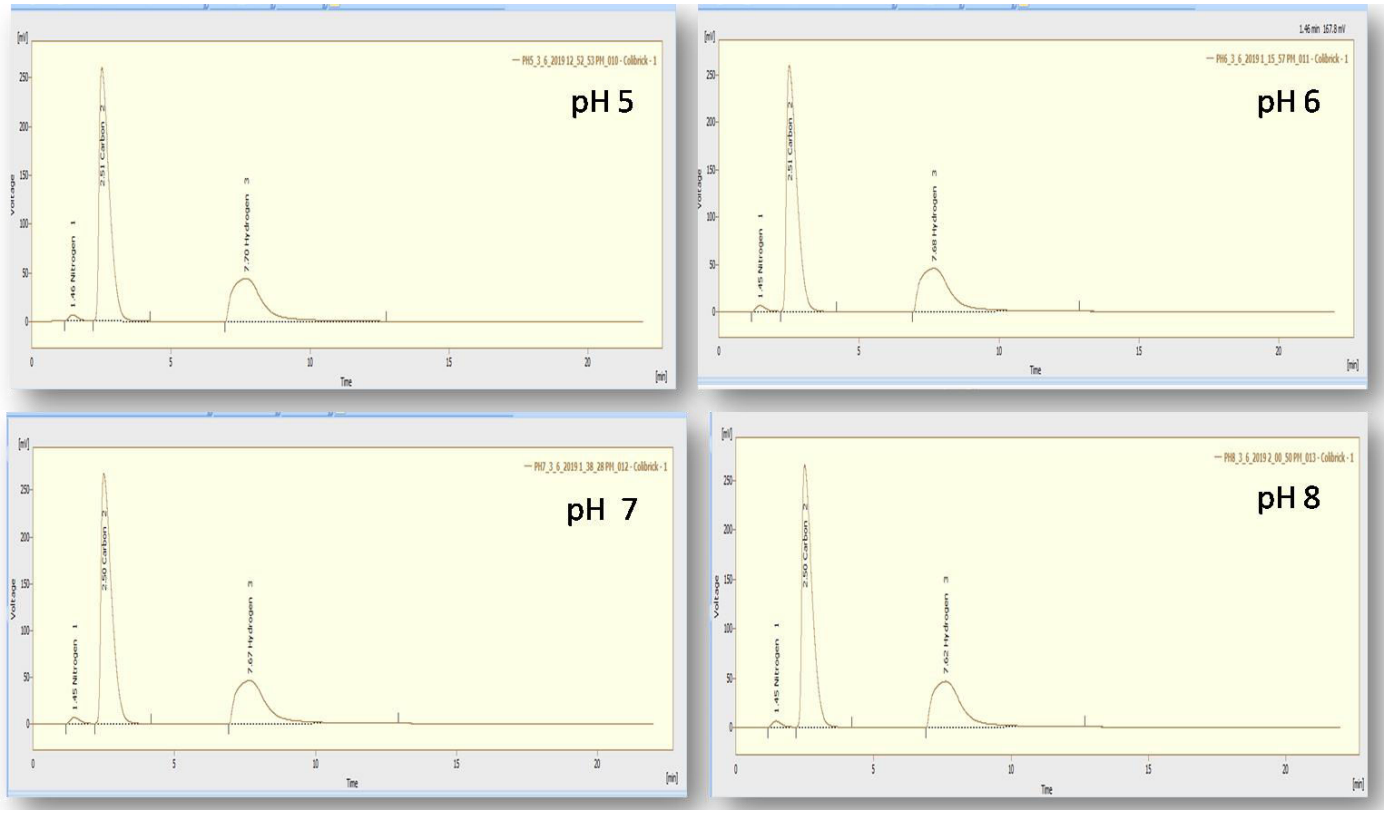

Figure 12: CHNS chromatograms for cordycepin quantification at different pH

4.5 EFFECT OF DIFFERENT TYPES OF SUBSTRATE

Substrate type plays an important role in cordycepin production. The highest cordycepin production was obtained in Brown Rice in both solid state fermentation and submerged fermentation. The cordycepin amount obtained was 148.93 mg and 141.76 mg in SmF and SSF condition respectively with brown rice as substrate.

Substrate	Cordycepin (mg)		Protein	
	SmF	SSF	SmF	SSF
Bajra	55.91	113.52	329.5	
Wheat	28.32	50.03	212.7	
Corn	33.31	141.48	184.7	

Rice	62.21	43.72	590.5
Brown Rice	148.93	141.76	596
Sprouts	--	74.89	
Brown rice+ wheat	125.74		429.5

Table 13: Effect of different substrates in different conditions

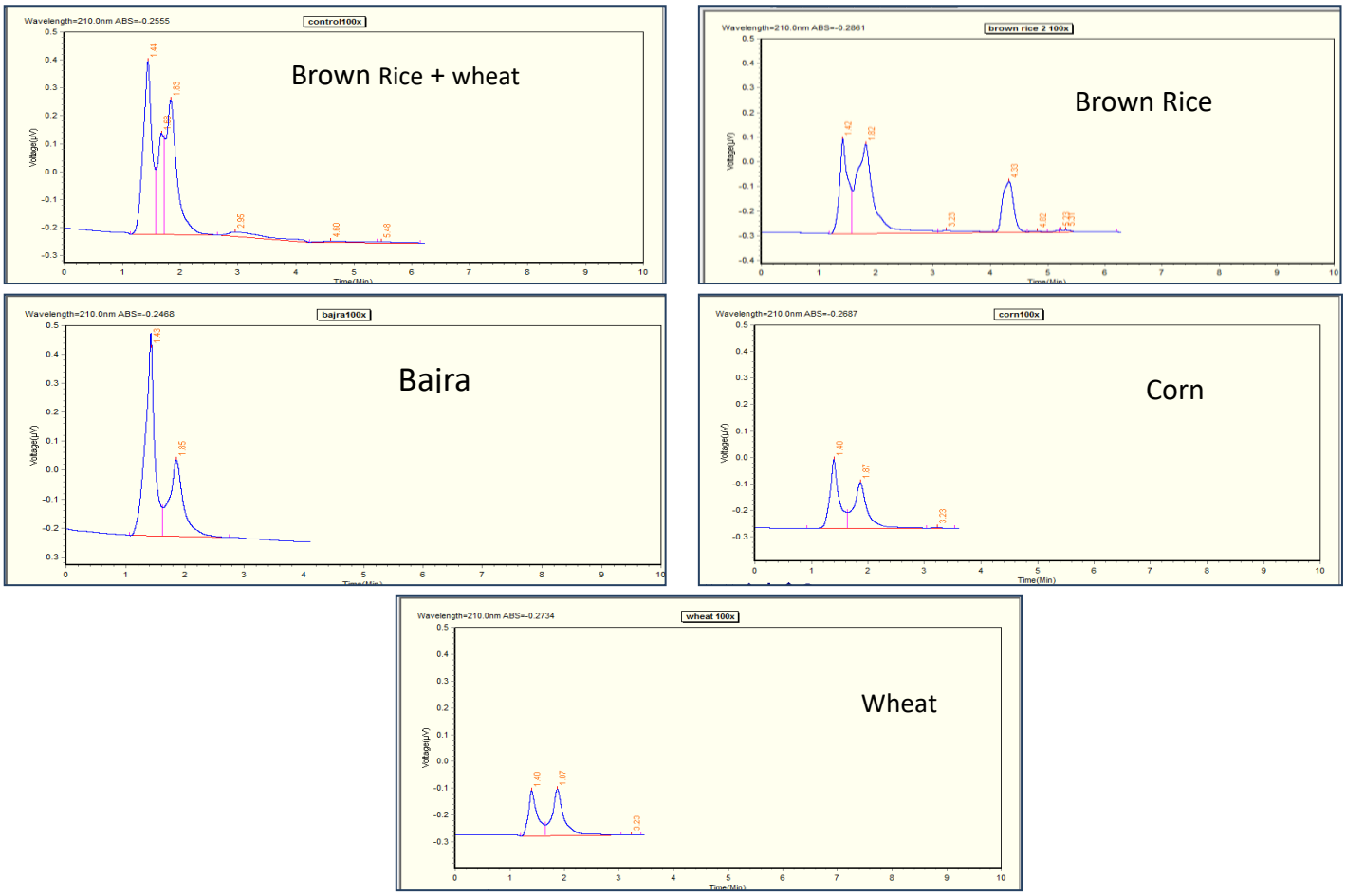

Figure 13: HPLC chromatograms for cordycepin quantification for different substrate kept in LSF

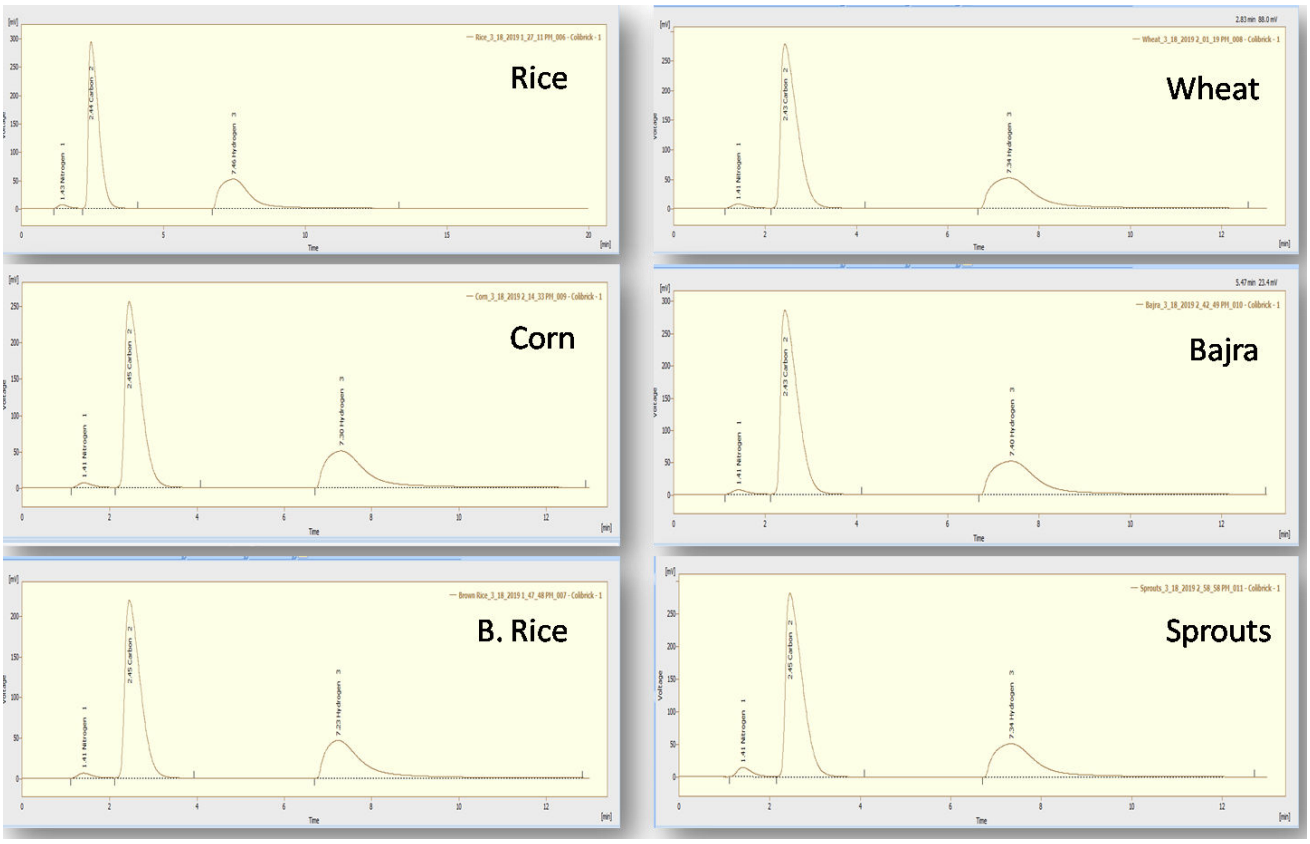

Figure 13: CHNS chromatograms for cordycepin quantification for different substrates

4.6 EFFECT OF LIGHT DURATION:

3 different light conditions were taken into consideration.

The first condition considered was exposure to light for 8 hours in a day.

pH	Cordycepin (mg)			Protein		
	SmF	LSF		SSF	SmF	LSF
		Mat	Broth			
5	175.44	19.52	44.59	67.97	385.2	
6	61.52	19.97	46.92	66.5	149.7	
7	17.55	18.5	43.72	73.24	378.7	
8	7.08	17.31	47.46	70.21	175.3	

Table 14: Effect of 8/16 light duration at different pH

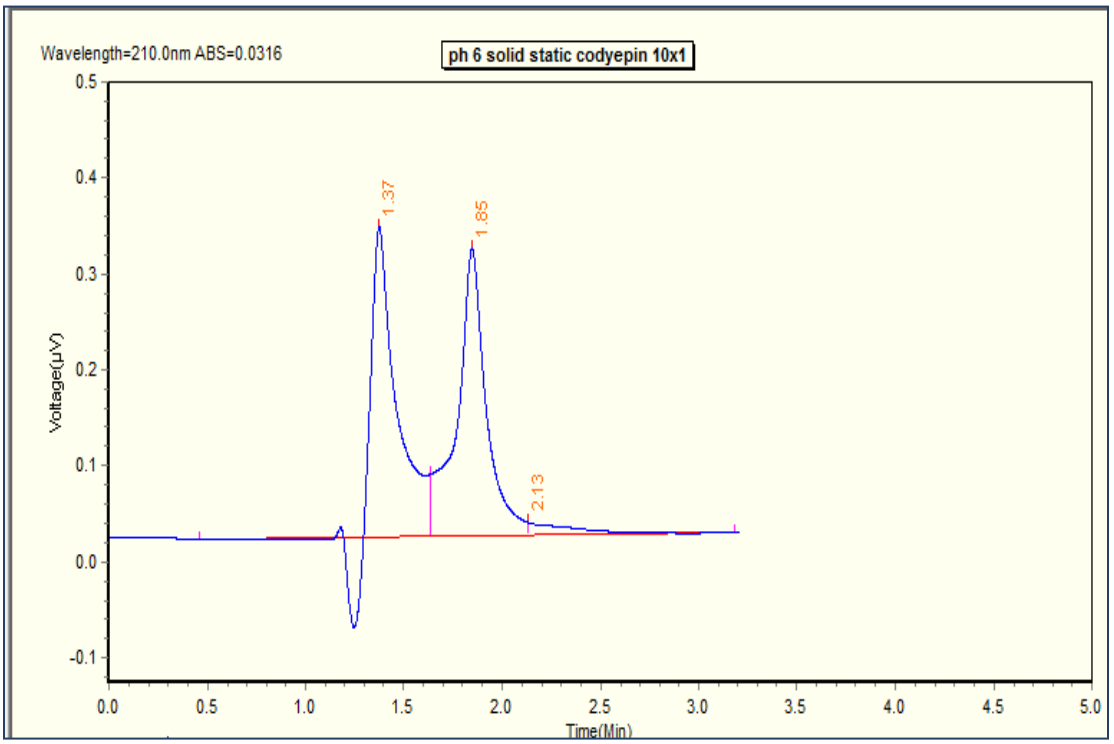

Figure 14: HPLC chromatograms for cordycepin quantification in Solid Static condition with 8 hours of light exposure .

The second condition considered was exposure to light for 16 hours in a day -

Substrate	Cordycepin (mg)		Protein	
-	SmF	SSF	SmF	SSF
Bajra	55.91	113.52	329.5	
Wheat	28.32	50.03	212.7	
Corn	33.31	141.48	184.7	
Rice	62.21	43.72	590.5	
Brown Rice	148.93	141.76	596	
Sprouts	--	74.89		
Brown rice+ wheat	125.74		429.5	

Table 15: Effect of different substrates in different conditions

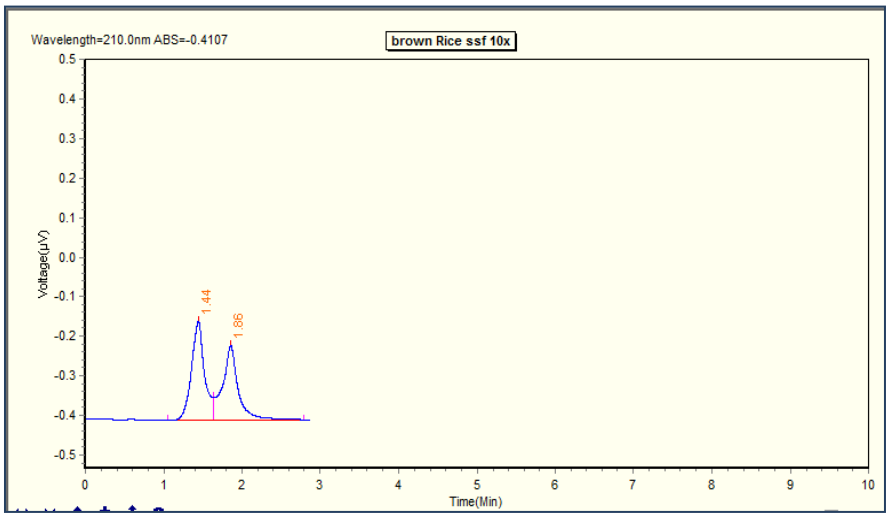

Figure 16: HPLC chromatograms for cordycepin quantification with 16 hours of exposure to light

4.7 EFFECT OF DIFFERENT SUBSTRATE CONCENTRATION

Substrate concentration had significant effect on cordycepin amount extracted. The cordycepin amount extracted when with 15g substrate was 66.50 mg and with 25 mg substrate it was 87.75 mg.

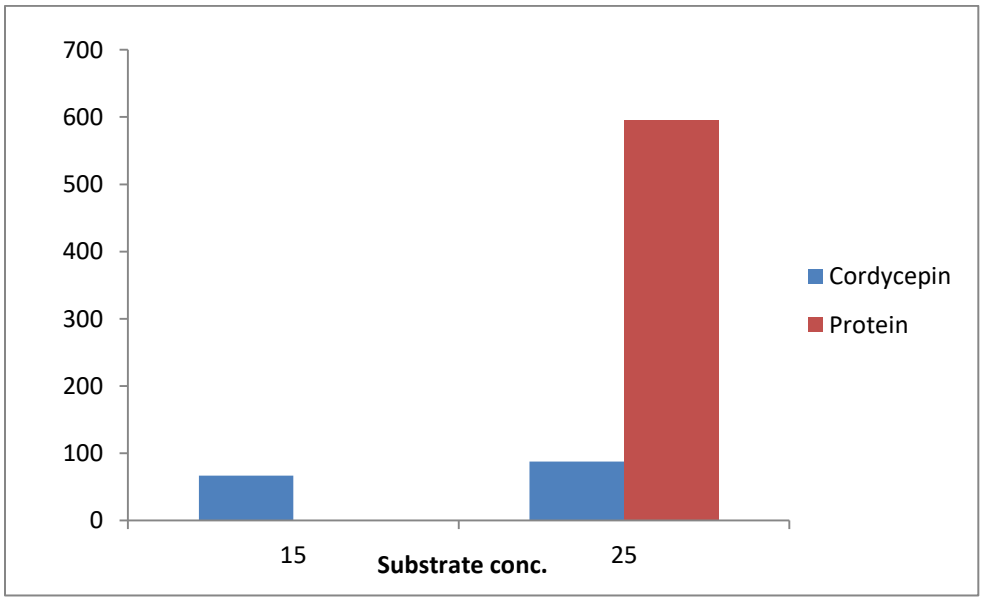

Figure 17: Effect of substrate concentration

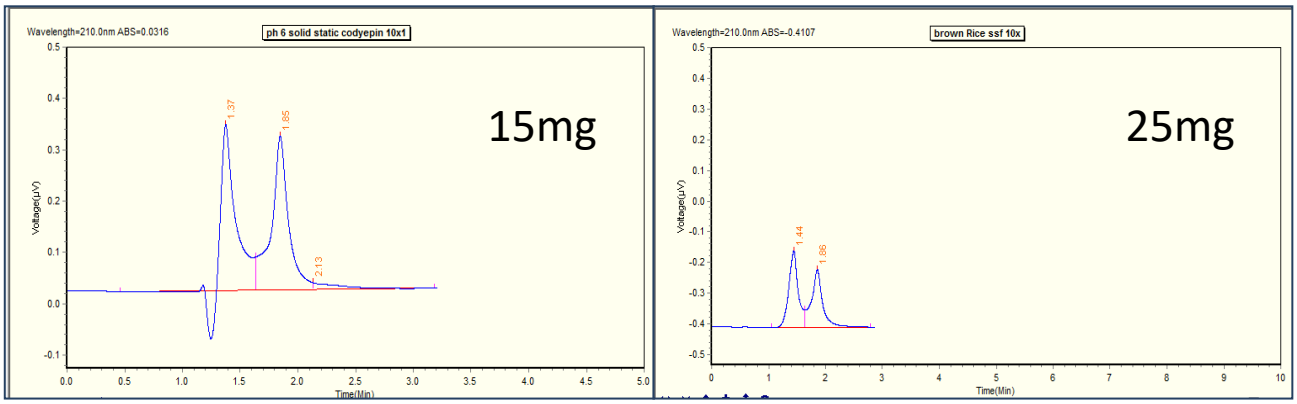

Figure 18: Effect of substrate concentration on cordycepin production

4.8 PROTIEN ESTIMATION

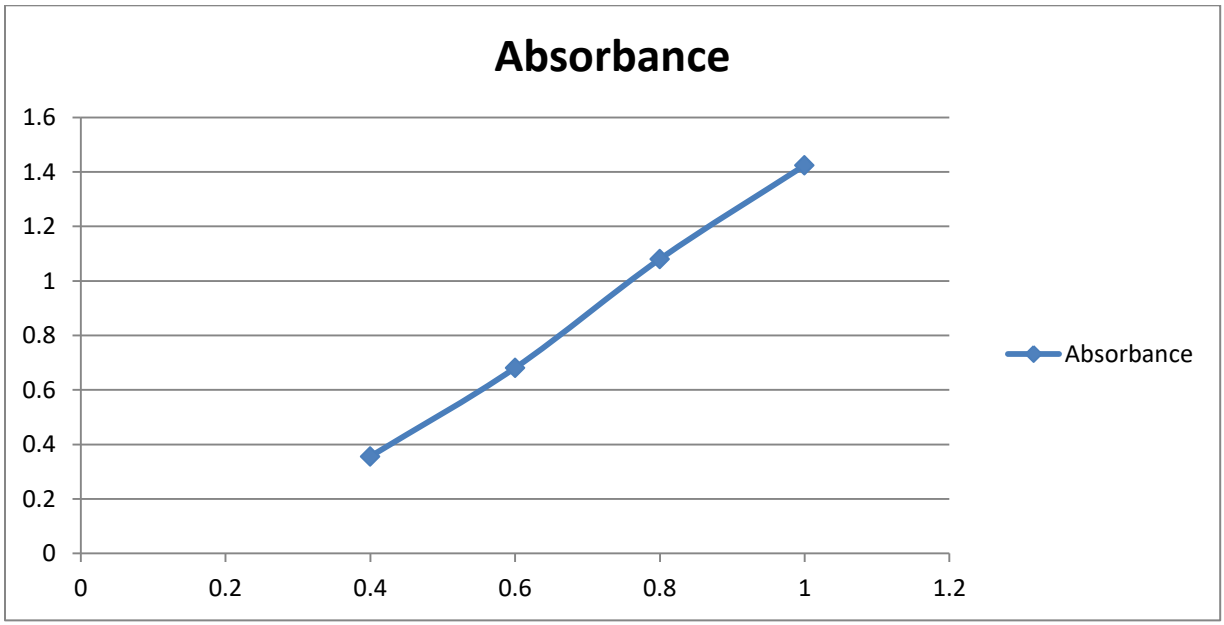

Figure 19: Standard curve for protein estimation

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