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Sequential optimization of *aspergillus flavus* kla-80 for the production of naringinase using stastical experimental designs

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Key words: Naringin, Naringenin, *Aspergillus flavus*, Response surface methodology, Central composite design

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Abstract

Naringin is a flavanone bitter glycoside which is found in flowers and fruit of the grapefruit, which on further hyrdolysis yields naringenin and glucose. As naringin is responsible for the bitterness of the fruit, to neutralize the bitterness by eco-freindly method, microbial method is performed by using naringinase enzyme. In the presesnt study naringinase producing strain *Apergills flavus* KLA-80 which was previously isolated from soil sample of Kalyan-Karnataka region, India, and deposited in NFCCI with an accesion number of OQ152018 which was maintained in the lab conditions was used for optimization studies.Further to increase the activity of enzyme one factor at a time approach was carried out where the activity was found to be increased from 559 to 720U/mL in a temp of 35°C, pH 4.5, carbon source 1.25% and nitrogen source of 0.15% at an agitation speed of 120rpm repectively. Eventually the production of naringinase from *Aspergillus niger* is optimized by Response Surface Methodology i.e., by CCD (Central Composite Design) method considering four factors at a time (pH,Agitation speed, Galactose, Peptone) as the variables. Based on the statstical method of optimization it is found that the enzyme activity was increased upto 20.41%.

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Introduction

In the present scenario, fruit juices are gaining importance because of their freshness and its property to increase the immunity of the body. Out of many major types, citrus family fruits are known to be one of the world famous crop as they are rich in particular vitamins, minerals and bioactive compounds. In India, different varieties of citrus fruits grown including mosambi, kinnow, oranges as they belongs to the family of Rutaceae and Plantae kingdom (Tran *et al.,* 2020). As the production of citrus fruit stands at 3rd position after Bannana and Mangao as per the report of National Horticulture for the 2nd advance estimate of the year 2019-20. As these cirtus fruits are rich in phytochemicals which mainly contains naturally occurring biological active compounds including lipids, phenols, alkolids, diterpens etc (Mishra and kar, 2003). Flavanoids are the know to be a major secondary metabolities of the plants (Chen *et al.,* 2016) as These flavanoids are found to have a natural anti-oxidant properties which surge the body's defense mechanism, which was found to be one of the riverting hail in the recent medicine (Adami *et al.,* 2022). A precise of around 4000 flavonoids has known to be discovered in fruits, herbs and vegetables (Cook 1996). Among these flavnoids, Naringin is a flavanone glycoside which is known to present in citrus and grape fruits, as it bears a recognizable bitter taste to the fruits (Alam *et al.,* 2014). It is a compratively water soluble bitter compound present in fruit membranes namely Albedo and predominatly present in peels followed by seeds (Manish and Veeya, 2022). As these bitterness inturn causes the reduction to quality and the commercial value of the fruits thereby making it as a presistent problem in recent times (Zhang *et al.,* 2022).To make citrus fruit for consumer acceptibilty, the bitterness should be reduced below its threshold level by various physio-chemical methods which has been flourished (Munoz *et al.,* 2022). In physio-chemical method various different absorbents has been tested in which Synthetic natural resin showed highest efficieny (Riberio *et al.,* 2002) and also in event to this ion-exchange resins are also showed reduction in bitterness thereby maintaing the taste and acidity of the juice (Kranz *et al.,* 2010). But one of the drawbacks of these methods is that it alter the chemical reactions which leads to the mislaying of certain nutrients and also because of it's prohibitive costs which are not recomandable nowadays (Lee and kim, 2003). In order to overcome the drawbacks of these physico-chemical methods, Biological method of conversion of bitter compound is scruntinized i.e., by enzymatic method using naringinase enzyme which has a multi-enzyme activity. (Puri M, 2011). Naringinase was also used in the enhancement of aroma in wine and also in the prepararion of natural glycoside hydrolysed product (Puri *et al.,* 2009).

In order to optimize the enzyme production, classical methods which are used in the early days are found to be a tedious time consuming and also not recomended if larger number of varibales are involved (Sandom 1958).In this regard to enhnace the production optimization of the media is done by RSM method, which is a powerfull and efficient mathematical approach applied for the optimization of fermentation process which also includes medium optimization for enzyme production (Dutt *et al.,* 2009). CCD method is one of the integral part of RSM. As it is a set of statistical techniques and mathematical tools which is applied in the progression of appropriate functional relationship between the response and relatable input variables (Amiri *et al.,* 2019).

As this method has several advantages like reducing the number of experiments, predicting the response, checking the adequacy of the method, requries shorter time and more economical compared to the classical method (Oskouie *et al.,* 2008). It also helps to study the interaction effects between the parameters and also in determining the optimum conditions are the min advnatages of the RSM (Jitolis *et al.,* 2021).

In the present investigation, submerged fermentation of *Aspergillus flavus* KLA-80 was done for the production of naringinase by one factor at a time method of optimization follwed by Cental Composite Desgin where the synchronous effect of 4 different variables were analysed for the optimal naringinase production.

Materials and methods

Micro-Organism and Culture Media

An isolate *Aspergillus Flavus* KLA-80 was maintained in our laboratory at 4°C, which was investigated in this study for the naringianse production, was isolated from the soil of Kalyan-Karnataka region (India) which is identified and deposited in the NFCCI, and accession number of OQ152018 is obtained. The strain was maintianed in the agar plate containing (g/l^{-1}) following components with slight changes as prescribed by (Mukund *et al.* 2013) NH_4NO_3,5gm; Kcl,0.2gm; KH_2PO_4,0.4gm; $FeSO_4.7H_2O$,0.01gm; $ZnSO_4$,0.01gm; $MnSO_4$,0.01,gm; $MgSO_4$,0.2gm; Agar,15gm; Naringin,1gm; at pH 4.5.

Preparation of spore suspension

The spore suspension of the isolated culture KLA-80 was developed from the 7 days old culture grown on Naringin Agar Media in a 10ml of serile water containing 0.01% of Tween-80 by suspending the spores with a sterile loop (Shivalee *et al.,* 2018).

Quantitaive essay for naringinase activity

The activity of Naringinase was done by the method as prescribed by (Davis 1947)with some modifications.The fermented broth was allowed to centrifuge at 3000 rpm for 10min using Cryogen Refrigerator centrifuge and the 0.1ul of supernatant was allowed to mix with 0.9ul of naringin (0.05% of naringin dissolved in 0.1M sodium acetate buffer pH-4) and this mixture is allowed to incubate at 50°C for 60min after incubation 0.1ul of incubated solution is added with 5ml of 90% of diethylene glycol and the 0.1ul of 4N NaOH is added to stop the reaction and the reaction was allowed to incubate at 20 min for the color development and finally, the $O.D_{420}$ of residual naringin was measured by using UV-Visible spectrophotometer. All the data was measured three times in parallel. One unit (U) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1µ/mol of naringin/ min at the assay conditions (Puri and Kalra, 2005).

Effect of physical parameters

Some of the parameters like (temperature, pH, agitation speed, inoculum size and age (days) were studied to find its influence on the naringianse production in the basal media used.

The effect of temperature on the basal media was assesed by the incubating the reaction mixture at different temperature such as 25,30,35,40,45,50. Naringinase activity was examined for every 24 hrs for the successive 5 days.

pH in the radius of 3-6 were investigated for their effect on basal media for the production of naringinase. The pH of the medium was adjusted using 1N Hcl or 1N NaoH. The flasks were incubated at 35°C for 5days. The samples are withdrawn regularly for the determination of the naringianse activity.

Effect of stirring was carried at different rpm to determine the activity of naringinase i.e at an agitation speed of of 0, 60, 80, 100, 120, 140, 160, 180 and 200rpm. The activity was determined at regular interval of time.

The fungi was fatten in the basal media and the sowing was carried out at different inoculum size ranging from 0.25-1.50ul/ml. The inoculated flasks were incubated at 35°C for 5 days, further the naringinase activity was investigated at the regular interval of time.

Naringinase activity was measured by incubating production media which was seeded with different inoculum age starting from 12hr, 18hr, 24hr upto 5 days. The activity was recorded at regular intervals. The experiments were carried in the 500ml of conical flask conatining 100ml of the desried medium (Mukesh *et al.,* 2012).

Effect of carbon and nitrogen sources

Numerous carbon sources such as glucose, lactose, rhamnose,sucrose, starch, maltose and galactose were evaluted to determine it effects on the narinignase production by supplementing them has a carbon source for the basal production media.

Different organic and in-organic sources choosen for the study were peptone, beef extract,yeast extract,

malt extract, $NaNO_3$ and NH_4NO_3. As thses nitrogen sources were selected to determine effect on naringinase either it will be having a negative or positive effect. The basal media was supplement with metal salts such as, LiSO4, FeSO4, CaCl2, KCl, NaCl, MnSO4 and ZnSO4 (Sukhvir *et al.,* 2016).

Experimental design and stastical analysiss

Optimization of cultural media for the enhanced production is always been a tedious tasks. To overcome such kind of interpretation, RSM method has been designed, in which CCD (Central Composite Design) is one of the integral part of the RSM. In which four independent factors selected for naringinase production are agitation speed, carbon sources, nitrogen sources and pH. As these individual independent variables has two levels -1 and +1. The factors were set at four different variables coding levels of the factors refer to transforming the analyzed real value into co-ordinates inside.The experimental design includes a set of 30 experiments. The analysis of variance (ANOVA) was applied to determine the effect of inoculum percentage and regression analysis to predict naringinase activity. The conseques of all terms in the polynomial were judged analytically by computing the F-value at a probability(p) of 0.001,0.01 or 0.05. The regression co-efficients were then used to make stastical calculation to generate contour maps from the regression models (Lee *et al.,* 2006).

Results and discussion

Production of naringinase from microbial source and making it economically cheap to for the use of industries has been a worth praising achievement in the field of fermentation technology.

Physical parameters

Environmental conditions have its effect on the production of extracellular enzyme. Some physical parameters like temperature, pH, agitation speed, inoculum size and age were studied for investigating their effects on the naringinase production.

As mentioned in the below Table1, The optimal incubation temperature for the production of naringinase was found to be 35°C as this result was expected since the tested isolate is a mesophilic in nature. A slight decrease in an enzyme production was observed thereafter at 40°C and so on. In relation to this most of the isolated micro-organisms showed optimal naringinase production in the range of 25 to 40°C. It reported that *Aspergillus niger van Tieghem MTCC 2425* showed optimum activity at 29°C (Borkar *et al.,* 2020).Whereas *Aspergillus tubingensis* UA13 produces siginficant amount of naringinase at 35°C (Xia *et al.,* 2021). High temperatures also been reported to produce narainginase as reported by *Aspergillus oryzae* 11250 which shows optimal temperature of 45°C (Zhu *et al.,* 2017) respectively

Nearly all the naringinase isolated till now has found to have pH in the range of 4-6. Various pH ranging from 3-6 were tested in this investigation, were the pH 4.5 was found to produce highest naringinase activity as mentioned in below table 1, when compared to other pH.

The naringinase produced from *Bacillus methylotrophicus* (Mukund *et al.,* 2013) at pH 6 was found to produce highest naringinase activity of 8U/L. At pH 4.7 *Aspergillus niger* 2425 (Borkar *et al.,* 2020) found an optimal activity for the naringinase production. On the other hand Immobilized Naringinase isolated from *Aspergillus niger* also showed optimum activity at the pH4 (Gupta *et al.,* 2021).

Agitation leads to better movement of oxygen and mass transfer in different phases as it is also konwn to balance homogenous physical and chemical envirnoment in the media by ceaseless stirring. In the present investigation isolated naringinase showed an highest activity at 120rpm.Further agitation of 150rpm showed highest enzyme activity for the enzyme isolated from the novel strain *Bacillus methylotophics* (Mukund *et al.,* 2013). The naringinase isolated from *Aspergillus niger VB07* (Vinothkumar *et al.,* 2010) showed highest activity of 17.12IU/ml at an agitatio speed of 180rpm. The bacteria *Staphylococcus xylosus* MAK2 (Puri *et al.,* 2010) showed highest activity of 8.9IU/ml at an agitation speed of 300rpm.

Inoculum size plays an crucial role in fermentation process as it effects cultural parameters such as growth rate, nutritional utilization and cultural morphology. In this ongoing investigation the isolated strain KLA-80 was cultured in a basal nutrient media with different inoculum size. From the results it is found that naringinase activity gradually increases with increasing its inoculum upto reaching its maximum at an range of 0.25-0.75%. *Aspergillus niger* isolated from rotten pomelo (*citrus maxima*) found to produce highest activity at 0.5% of inoculum size (Sanjay *et al.,* 2020). Whereas naringinase produced from using Amla (Supriya chalta *et al.,* 2022). as substrated showed highest activity of 111870.3U/gm at an inoculum size of 20%.

In case of inoculum age, as this plays a crucial role while using mesophilic micro-organisms due to relatively low growth rate.In the present investigation different inoculum age is tested i.e from 24hrs to 8 days. It is found that highest naringinase activity was observed at 5th day of incubation. *Rhizophus stolonifer* using paddy husk showed highest activity of 1073.97u/g after optimization of the basal medium on the 8th day (Karuppaija *et al.,* 2016). Whereas *Aspergillus niger* MTCC 1344(Puri *et al.,* 2005) showed highest naringinase activity of 968 u/g on the7th day of fermentation.

Table 1. Physical parmaters in relation to enzyme activity.

SL	Parameters	Range	Activity (Increment)
1	pH (4.5)	3.5-6	610
2	Temp (35)	30-55	630
3	Agitation speed (180)	60-180	651
4	Inoculum size (0.75)	0.25-0.50%	668
5	Inoculum age (5^{th} day)	24hrs- 7days	685

One factor at a- Time-method

In the present study, after 5 days of fermentation, galactose showed highest naringianse activity when compared to other carbon sources like glucose, lactose, rhamnose, sucrose, starch and maltose. Hence galactose (700U/mL at 1.25%) is found to be a one of the cheap resources and also doesnot have any demerits, it was further selected for media formulations in subsequent experiments. Eventually, a trial was conducted to determine the optimum nitrogen sources required for the narinignase production. A total of 6 different nitogen sources are studied in this investigation which includes peptone, beef extract, yeast extract, malt extract, $NaNO_3$ and NH_4NO_3. Among all these 6 nitrogen sources, peptone (752U/mL at 0.15%) was found to be most effective and notably superior than other sources. In this view, it is found that organic nitrogen sources yield higher naringinase activity then in-organic nitrogen sources in sub-merged fermentation. As in the case of *Bacillus methylotrophicus* sucrose (1%) along with yeast extract (0.5%) showed highest activity of 12.05U/L when compared to the basal media (Mukund *et al.,* 2013). In the case of Rhizopus stolonifer 1.5% of glucose concentration and 0.5% of ammonium nitrate produced a high amount of naringinase i.e 1073.97U/g which is expentionally more then the unoptimized media (Karuppaija *et al.,* 2016). Whereas Aspergillus niger isolated from the soil showed an increase in activity upto 1.8 times with an 0.5% rhamnose and 0.25% peptone (Vinothkumar *et al.,* 2010).

Metal ions are acts as stimulator for the growth of micro-organsims as its in co-operation in the media is neccessary as it effects the enzyme production. In the present investigation, metal ions of concentration 0.05% is used where Mg^{2+} showed highest activity of 760U/mL whereas Mg^{2+} is followed by $Cu^{2+}>Mn^{2+}>Zn^{2+}$. In case of *Aspergillus Flavus* Isolated From Decaying Citrus Maxima Fruits showed realtive activity of 133.913% at an 0.1% of Na^{2+} concentration(Srikantha *et al.,* 2017)

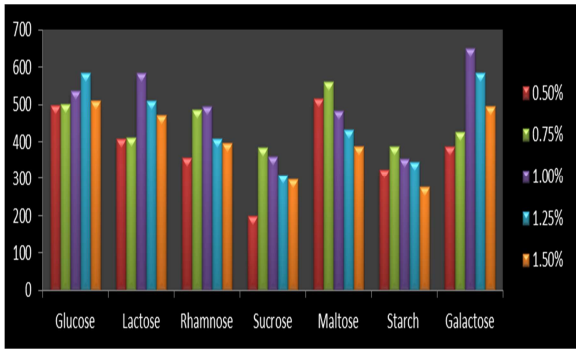

Fig. 1. Naringinase activity recorded for various combinations of carbon source (0.25-1.50%).

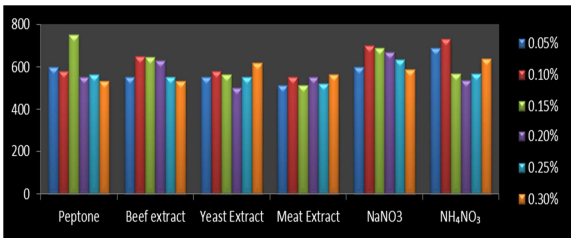

Fig. 2. Naringinase activity recorded for various combinations of nitrogen source (0.05-030%).

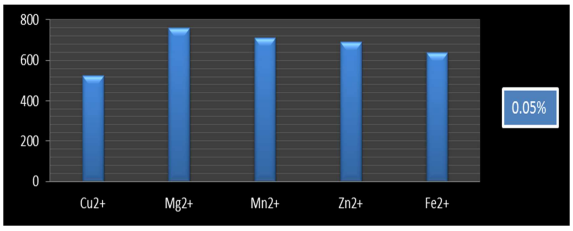

Fig. 3. Naringinase activity recorded for various combinations of metal ions (0.05%).

RSM

Subsequently optimizing the values of variables by "One-factor-at-a-time-method", 4 most importnant varibales were selected for Response Surface Methodology by CCD method.

Response surface methdology is know to be one of the recomended staistical method which is used to optimize the optimum level of fermentation conditions which helps to analyze the infulence of several variables at a time along with the estimation of thier interaction on the production of naringinase. The infulence of 4 process parameters for fermentation was analyzed by Central Composoite Design with 4 factors(Kalil *et al.,* 2000). The design of the experiment are represented in below Table 2.

Table 2. Design of RSM.

Std	Run	Factor 1 A:pH	Factor 2 B: Agitation spe... rpm	Factor 3 C: Galactose %	Factor 4 D: Peptone %	Response 1 Naringinase U/mL
24	1	4.75	120	0.875	0.425	775
27	2	4.75	120	0.875	0.175	867
8	3	6	180	1.5	0.05	589
26	4	4.75	120	0.875	0.175	867
9	5	3.5	60	0.25	0.3	700
5	6	3.5	60	1.5	0.05	538
6	7	6	60	1.5	0.05	521
21	8	4.75	120	-0.375	0.175	812
1	9	3.5	60	0.25	0.05	579
13	10	3.5	60	1.5	0.3	619
20	11	4.75	240	0.875	0.175	648
23	12	4.75	120	0.875	-0.075	651
19	13	4.75	0	0.875	0.175	618
10	14	6	60	0.25	0.3	766
15	15	3.5	180	1.5	0.3	593
18	16	7.25	120	0.875	0.175	433
4	17	6	180	0.25	0.05	646
17	18	2.25	120	0.875	0.175	408
25	19	4.75	120	0.875	0.175	867
22	20	4.75	120	2.125	0.175	686
12	21	6	180	0.25	0.3	654
14	22	6	60	1.5	0.3	613
16	23	6	180	1.5	0.3	582
7	24	3.5	180	1.5	0.05	619
2	25	6	60	0.25	0.05	611
11	26	3.5	180	0.25	0.3	644
29	27	4.75	120	0.875	0.175	867
3	28	3.5	180	0.25	0.05	621
28	29	4.75	120	0.875	0.175	867

The consequence of the model was analyzed by ANOVA method. The tools used for the determination of significance were *F* value and *p* value and it is also used to understand relationship between process variable and co-efficient. If the *p* value is smaller and F value is higher than the corresponding co-efficient (Mukund *et al.,* 2013). Then the *F*- value was computated as the mean square of residual and the

mean square of regression and if the *p* value is less than 0.05 indicates that the terms used for the model are significant, depending upon the importance of particular variable in the study.

Table 3. ANOVA Table.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	4.454E+05	14	31817.30	292.69	< 0.0001	significant
A-Ph	590.04	1	590.04	5.43	0.0353	
B-Agitation speed	155.04	1	155.04	1.43	0.2522	
C-Galactose	26600.04	1	26600.04	244.69	< 0.0001	
D-Peptone	20126.04	1	20126.04	185.14	< 0.0001	
AB	410.06	1	410.06	3.77	0.0725	
AC	2425.56	1	2425.56	22.31	0.0003	
AD	150.06	1	150.06	1.38	0.2596	
BC	2093.06	1	2093.06	19.25	0.0006	
BD	12712.56	1	12712.56	116.94	< 0.0001	
CD	1743.06	1	1743.06	16.03	0.0013	
A^2	3.334E+05	1	3.334E+05	3067.34	< 0.0001	
B^2	94152.84	1	94152.84	866.11	< 0.0001	
C^2	25320.95	1	25320.95	232.93	< 0.0001	
D^2	42012.30	1	42012.30	386.47	< 0.0001	
Residual	1521.92	14	108.71			
Lack of Fit	1521.92	10	152.19			Not significant
Pure Error	0.0000	4	0.0000			
Cor Total	4.470E+05	28				

The model F-value of 292.69 and the p-value less than 0.0001 implied that model was significant and there is only 0.01% chance that the mode F-value could occur due to noise. For the good statistical model R^2 value should be close to 1.0. Adequate precision measure the signal to noise of ratio, a ratio of 61.74 indicates adequate signal. The value of the adjusted determination co-efficient (Adj R^2 = 0.9932) was also high to advocate for the high significance of the model. All these considerations indicate good adequacy of the regression model.

The 3 dimensional diagram and plots are given by regression model are plotted below in the fig. 4. The 3 dimensional images is obtained in a combination i.e. 2 variables in a centre and 2 variables are independent. From the curve of three-dimensional plots optimum concentration of the media is recognized. The counterpart pinnacles the character played variables and their interactive effect. From the fig 4, it is observed that increase in the variable concentration increases the naringinase activity upto an optimal level and there after significant decrease in naringinase activity is observed. The counter plots showed a broad plateau region in which the activities change relatively little when there is concentration change in the nutrient is observed. Thus it is observed that optimal solution concentration can accommodate small errors.

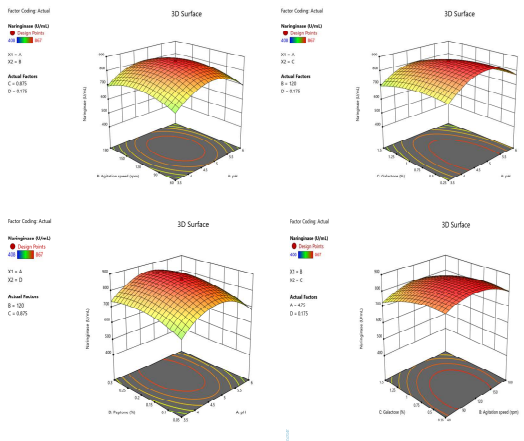

Fig. 4. Three-dimensional plot of Naringinase activity of *Aspergillus flavus* KLA-80 a) agitation speed and pH. b) Galactose and pH. c) Peptone and pH. d) Galactose and agitation speed.

For the determination of optimum values of the variable for the maximum production of naringinase point prediction method was used as per the equation given by (Mukund *et al.,* 2013).In the present investigation galactose of 1.25%, peptone-0.15%, pH of 4.75 and agitation speed of 120rpm showed highest

activity of 867 U/mL i.e 20% increment in the activity is observed when compared with the activity obtained by one-factor-at- a-time method.

Conclusion

Naringinase was known to be one of the important enzymes as it converts bitter naringin to no-bitter naringenin. As this enzyme is one the budding approaches for the food industries. In this research previously isolated *Aspergillus flavus* KLA-80 were taken to optimize the production of naringinase. As this isolate produce 559U/mL. It is further optimized by one-factor-at-a-time followed by statistical experimental design was applied by RSM CCD method and there 20.41% is increase in the activity of the enzyme was observed i.e the activity increased to 780U/mL from 559U/mL at an galactose of 1.25%, peptone of 0.15%, naringin of 0.1%, agitation speed of 120rpm, pH of 4.75 and temperature of 35°C.

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Conflicts of interest

The authors report no financial or any other conflicts of interest in this work

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